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(54) Title: NUCLEIC ACID PROBES AND USES THEREOF TO DETECT DOUBLE-STRANDED NUCLEIC ACIDS

(57) Abstract

Processes and probe populations that allow efficient hybridization of probes to both strands of a two-stranded nucleic acid target without substantial self-hybridization within the probe population.

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NUCLEIC ACID PROBES AND USES THEREOF TO DETECT DOUBLE-STRANDED NUCLEIC ACIDS

FIELD OF THE INVENTION

This inventions described herein concern the detection of nucleic acids through the use of nucleic acid probes.

BACKGROUND

By the use of specific nucleic acid (RNA or DNA) probes, nucleic acid molecules that signify infection and other disease states may be detected. Certain genetic diseases are characterized by the presence of genes which are not present in normal tissue. Other diseased conditions are characterized by the expression of RNAs or RNA translation products (i.e. peptides or proteins) which are not expressed in normal cells. Some disease states are characterized by the absence of certain genes or gene portions, or the absence or alteration of expression of gene products or proteins.

When the probe target is DNA, the target is generally a two-stranded target: an "anti-sense" strand from which RNA such as mRNA is transcribed and a "sense strand" that is complementary in base sequence to the anti-sense strand. This suggests that, for a given amount of cellular DNA target, one can double the signal generated by target-bound probes by including both probes against the sense strand and the anti-sense strand of the target.

Generally speaking, however, there is a tendency for the efficiency of hybridization (amount of target-bound probe per ug of probe added to the assay) to decrease if the probe population contains not only molecules complementary to one strand of a target but also molecules complementary to the other strand of that target. This is particularly true as one increases the total probe concentration in an effort to achieve target

saturation in as short a time as possible. This decrease in efficiency is almost certainly due to the tendency of the probe molecules to hybridize to each other, thereby creating high molecular weight aggregates. In the present invention, appropriate measures are taken so that there is little or no decrease in the efficiency of hybridization when the probe population has probes against the sense strand and probes against the anti-sense strand.

BRIEF SUMMARY OF THE INVENTION

In the present invention, a nucleic acid probe population is created so that although some of the probe molecules are capable of hybridizing to one strand of a double-stranded target and other probe molecules are capable of hybridizing to the other strand of that target, the probe molecules cannot hybridize to each other. Optimally, one has a series of probes that can, on each strand of the target, hybridize in end-to-end fashion. Self-hybridization of the probe population is avoided, by limiting the size of the probe molecules and by limiting the length of the regions that are complementary between any two probe molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 Nucleotides 101 to 400 of Gen Bank sequence HUMREPA84 are marked and are shown such that nucleotide 101 is at the 5' end of the sequence and nucleotide 400 is at the 3' end. Nucleotides in the sequence complementary to that between nucleotides 101 and 400 are shown below those latter nucleotides. The nucleotide sequences of five probes, H18-100L (SEQ ID NO:16), H18-100R (SEQ ID NO:17), H18-110R (SEQ ID NO:18), H18-10 (SEQ ID NO:14), and H18-11 (SEQ ID NO:18), are indicated.

Fig. 2. Diagram showing relationships among probes used in Example 3.

Fig 3a. Photomicrograph of results obtained with probe HYR-7-25 (SEQ ID NO:19) in Example 4.

Fig. 3b. Photomicrograph of results obtained with probe HYR-7-12 (SEQ ID NO:21) in Example 4.

DETAILED DESCRIPTION

Consider that, for any pair of single-stranded molecules, if one has within itself a nucleotide sequence that is complementary to a nucleotide sequence in the second molecule, and both of those sequences are N nucleotides long (the total length of either molecule can be greater than N) then the molecules will form a hybrid only if N is larger than some critical value. The critical value will depending partly on the hybridization conditions (temperature, choice of solvent, etc.) and partly on the nucleotide composition of the complementary sequences.

The critical value in the experiments exemplified in the Examples below will be seen to be between 12 an 23. In those Examples, by letting the length of the probe molecules be about 24 nucleotides, and by not letting N exceed 12 for any pair of probe molecules, one has an effective probe population for hybridizing to a two-stranded target. The population is effective because one obtains about twice as much as signal as one would obtain with probes to just one strand.

By varying the hybridization conditions and/or the base sequences of the probe molecules, one can vary the critical value of N. This is evident from the Examples included here. Nevertheless, by routine experimentation according to the principles set forth below, one can determine the critical value for any set of hybridization conditions

and thereby perform the processes of this invention.

In one aspect, the invention is a process for detecting a two-stranded nucleic acid target, which process comprises the steps of:

(1) separating the strands of the target sufficiently to allow them each to hybridize to a nucleic acid probe of complementary nucleotide sequence;

(2) co-incubating the sufficiently separated strands of the target with a nucleic acid probe population that comprises molecules complementary in nucleotide sequence to one target strand and molecules complementary in nucleotide sequence to the other target strand; and

(3) detecting the nucleic acid probe molecules that are hybridized to target molecules;

such that step (2) is performed under conditions that allow each strand of the target to form a hybrid with a nucleic acid probe molecule complementary in nucleotide sequence to that strand;

such that, as to the nucleotide sequence of each nucleic acid probe molecule, there is a totally complementary sequence in the target;

such that each nucleic acid probe molecule is partially complementary in nucleotide sequence to at least one other nucleic acid probe molecule;

such that no two nucleic acid probe molecules are completely complementary in nucleotide sequence to each other;

such that, where a portion of one nucleic acid probe molecule is complementary in nucleotide sequence to another nucleic acid probe molecule, that portion has a length which is too short to allow it to hybridize to the other nucleic acid probe molecule under the conditions of step (2).

The term "nucleotide sequence" is intended to cover a sequence where there is some atom (e.g., sulfur) other than phosphorus at some of the positions where internucleoside phosphorus normally occur. In such a situation, one could alternatively two molecules complementary as to nucleotide sequence as being complementary as to nucleoside sequence or complementary as to nucleotide sequence.

The probe molecule will normally be labelled with a detectable label, e.g.,

radioactively (e.g. with ^{32}P), a dye molecule such as fluorescein, or a moiety that can enter into a chemiluminescence reaction.

In one general embodiment of the process, the two target strands are located in a biological entity that is either a cell or a virus. The cell or virus may be suspended in solution and not immobilized on a solid support. On the other hand, the cell or virus may be immobilized on a solid support. The cell or virus may be part of a tissue section (histologic section).

The cells containing the target nucleic acid molecules may be eukaryotic cells (e.g., human cells), prokaryotic cells (e.g., bacteria), plant cells, or any other type of cell. They can be simple eukaryotes such as yeast or derived from complex eukaryotes such as humans.

The target strands of nucleic acid may be in a non-enveloped virus or an enveloped virus (having a non-enveloped membrane such as a lipid protein membrane).

In one embodiment of the process, a plurality of molecules in the probe population are each covalently attached to a fluorescent dye molecule either directly or via a cross-linker molecule.

In the process, the two target strands may be purified nucleic acids. They may have been extracted from a virus, cell or multi-cellular organism.

The two target strands may be immobilized on a solid support (such as on nitrocellulose paper or a nylon sheet) during Step (2) of the process. Alternatively, they may be in solution and not immobilized on a solid support.

In the process, the target strands may be DNA. The target strands may be RNA, as in the case of a virus (e.g., human immunodeficiency virus) where complementary RNA strands can exist simultaneously in a single cell.

A viral nucleic acid target can be part of a virus, in which case the virus may or may not be inside a cell. Alternatively, a viral nucleic acid target may not be part of a virus, but may be inside a cell.

In a preferred embodiment of the process, the probe molecules have nucleotide sequences such that, if one strand of the target strand is saturated with probe molecules, then there will be no unhybridized target strand sequences forming gaps between the

probe molecules.

It is preferred that each probe molecule is complementary to a sequence, present in at least one other probe molecule, not less than about 12 nucleotides but not more than about 100 nucleotides in length.

More preferably, each probe molecule is complementary to a sequence, present in at least one other probe molecule, not less than about 12 nucleotides but not more than about 20 nucleotides in length.

In one preferred embodiment, each probe molecule that is complementary to a sequence, present in at least one other probe molecule, that is about 12 nucleotides in length.

It is preferred that the length of each probe is between about 15 nucleotides and 100 nucleotides. It is more preferred that the length of each probe is between about 15 nucleotides and 40 nucleotides.

In the process, it is preferred that the portion of a probe molecule that is complementary to another probe molecule is not less than about 12 nucleotides but not more than about 100 nucleotides in length. It is more preferred that the portion of a probe molecule that is complementary to another probe molecule is not less than about 12 nucleotides but not more than about 20 nucleotides in length. In one highly preferred embodiment of the process, the portion of a probe molecule that is complementary to another probe molecule is about 12 nucleotides in length.

In particular embodiments of the above processes, the two-stranded target has a first target strand and a second target strand and wherein the probe molecules that are complementary in nucleotide sequence to the first target strand have a detectable label with a structure different from the detectable label on the probe molecules that complementary to the second target strand. For example, the detectable label on the probe molecules that are complementary in nucleotide sequence to the first target strand may be a fluorescent dye and the detectable label on the probe molecules that are complementary to the second strand may also be a fluorescent dye. In a particular embodiment, wherein the two-stranded target is a DNA target, the probe molecules that are complementary in nucleotide sequence to the first target strand are also

complementary in nucleotide sequence to cellular RNA molecules. An example of where the latter particular embodiment is useful is where there may be a double-stranded DNA viral genome (or the reverse transcriptase DNA copy of an RNA viral genome) in the target cell of interest and, if indeed there is such a genome present, then there may or may not be RNA transcribed from such a genome. Not only is it of interest, from a clinical point of view, to know whether the DNA genome is present, it is of clinical interest to know whether that genome is being expressed into mRNA or other RNA copies of the genome. If there is no viral mRNA (or other RNA) present, then the amount of nucleic acid detected by the probe against the anti-sense strand will equal the amount of nucleic acid detected by the probe against the sense strand. If there is also viral mRNA present, then the amount of nucleic acid detected by the probe against the sense strand of DNA will exceed the amount of nucleic acid detected by the probe against the anti-sense strand of DNA. The excess will be due to the mRNA present. Indeed by calibrating the probes against known amounts of viral RNA and viral DNA so that the amount of flourescence given off by a given amount of hybridized probe is known, one can use the results to calculate the amounts (total mass) of viral RNA and viral DNA present in a test sample and also, by considering the molecular weight of the RNA and DNA targets, calculate the number of copies of RNA molecules and number of copies of viral DNA per test sample and therefore per cell.

If the strategies of Examples 4 and 7 are followed using 30-mers with four fluors each, and if hybridization is done with cells on slides, and if enough 30-mers are used to cover both strands of a target, then one should be able to detect a single copy of the target in a single cell even if the target is as short as 750 base pairs and fluorescence is observed by eye with a microscope, or as short as 75 to 150 base pairs with an image analysis system.

The two-stranded target may be cellular DNA, cellular RNA, viral DNA, or viral RNA.

In addition to the various processes of the invention, the inventions here are also the nucleic acid probe populations, including all specific and preferred embodiments, disclosed here for use in those processes.

Related inventions are probe populations used in the above-noted process of the invention. An example is a nucleic acid probe population wherein

1) the length of each probe molecule is between about 15 nucleotides and about 100 nucleotides,

2) no probe molecule is totally complementary in nucleotide sequence to another probe molecule, and

3) each probe molecule is at least partially complementary in nucleotide sequence to at least one other probe molecule.

Probes

The nucleic acid probe may be DNA, RNA, or oligonucleotides or polynucleotides comprised of DNA or RNA. The DNA or RNA may be composed of the bases adenosine, uridine, thymidine, guanine, cytosine, or any natural or artificial chemical derivatives thereof. The probe is capable of binding to a complementary or mirror image target cellular genetic sequence through one or more types of chemical bonds, usually through hydrogen bond formation.

Nucleic acid probes may be detectably labeled prior to addition to the hybridization solution. Alternatively, a detectable label may be selected which binds to the hybridization product. Probes may be labeled with any detectable group for use in practicing the invention. Such detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see Clin. Chem., 22:1243 (1976)), enzyme substrates (see British Pat. Spec. 1,548,741), coenzymes (see U.S. Patents Nos. 4,230,797 and 4,238,565) and enzyme inhibitors (see U.S. Patent No. 4,134,792); fluorescers (see Clin. Chem., 25:353 (1979); chromophores; luminescers such as chemiluminescers and bioluminescers (see Clin. Chem., 25:512 (1979)); specifically bindable ligands; proximal interacting pairs; and radioisotopes such as ^{3}H, ^{35}S, ^{32}P, ^{125}I and ^{14}C.

The term "nucleic acid probe" is considered to include nucleic acids that have been

labeled in any manner, including the foregoing manners.

Biotin labeled nucleotides can be incorporated into DNA or RNA by nick translation, enzymatic, or chemical means. The biotinylated probes are detected after hybridization using avidin/strepavidin, fluorescent, enzymatic or colloidal gold conjugates. Nucleic acids may also be labeled with other fluorescent compounds, with immunodetectable fluorescent derivatives or with biotin analogues. Nucleic acids may also be labeled by means of attaching a protein. Nucleic acids cross-linked to radioactive or fluorescent histone HI, enzymes (alkaline phosphatase and peroxidases), or single-stranded binding (ssB) protein may also be used. To increase the sensitivity of detecting the colloidal gold or peroxidase products, a number of enhancement or amplification procedures using silver solutions may be used.

An indirect fluorescent immunocytochemical procedure may also be utilized (Rudkin and Stollar (1977) Nature 265: 472; Van Prooijen, et al (1982) Exp.Cell.Res. 141: 397). Polyclonal antibodies are raised against RNA-DNA hybrids by injecting animals with poly(rA)-poly(dT). DNA probes were hybridized to cells in situ and hybrids were detected by incubation with the antibody to RNA-DNA hybrids.

Photobiotin™ labeling of probes is preferable to biotin labeling.

Nucleic acid probes can be used against a variety of nucleic acid targets, viral, prokaryotic, and eukaryotic. The target for probe populations of this invention will usually be a DNA target such as a gene (e.g., oncogene), control element (e.g., promoter, repressor, or enhancer), or sequence coding for ribosomal RNA, transfer RNA, or RNase P. Alternatively, the target may be any nucleic acid target, either RNA or DNA that comprises one of the two complementary target nucleotide sequences; that will be the situation, for example, where the desire is to detect any DNA or mRNA molecule with a specific sequence or its complement. The target may be RNA where, as in the case of some viruses, a viral RNA sequence and its RNA complement may be present in the same cell.

As can be seen from the Examples, probes of any desired sequence can be made.

When the target is a purified nucleic acid

A purified nucleic acid is considered here to be one that has been extracted from a cell or has been synthesized in vitro in a cell-free system. Many procedures have been published for hybridizing probes to such purified nucleic acids. Generally, if the target is a DNA molecule, its strands are separated by heat or other means before the hybridization step takes place. The hybridization can take place with the target immobilized on a solid support (e.g., nitrocellulose paper for DNA, nylon for RNA) by well-established procedures. The probes may be labeled in the same way as probes are labeled for in situ experiments as described below; or they may be labeled in other detectable ways. The manner of labeling is not critical for implementation of this experiment. If a labeling procedure is known to work for probes against purified nucleic acid targets, it would be expected to work for probe populations where both strands are targeted.

Targets in cells, tissue, and fluids

The hybridization assay can be done for targets in biological entities in liquid suspension, in cells on slides or other solid supports, in tissue culture cells, and in tissue sections. When the biological entity is a cell, it can come from solid tissue (e.g., nerves, muscle, heart, skin, lungs, kidneys, pancreas, spleen, lymph nodes, testes, cervix, and brain) or cells present in membranes lining various tracts, conduits and cavities (such as the gastrointestinal tract, urinary tract, vas deferens, uterine cavity, uterine tube, vagina, respiratory tract, nasal cavity, oral cavity, pharynx, larynx, trachea, bronchi and lungs) or cells in an organism's fluids (e.g., urine, stomach fluid, sputum, blood and lymph fluid) or stool.

When the target is in a biological entity

Two very useful summaries of possible hybridization conditions are in PCT International Patent Applications with publication numbers WO 90/02173 and WO 90/02204, both of them applications of Research Development Corp.

In situ hybridization allows the detection of RNA or DNA sequences within individual cells. With sufficiently large targets, it can detect as few as 1-5 target molecules per cell in as little as 2-4 hours. (PCT Applications 90/02173 and Wo 90/02204) It also allows for the simultaneous detection of more than one different polynucleotide sequence in an individual cell. It also allows detection of proteins and polynucleotides in the same cell.

As noted above, many different hybridization conditions (solvent composition, temperature, time) are possible. The ones mentioned below are only intended to advise the reader of some of the more preferable hybridization conditions. As anyone skilled in the art will know, many other conditions could also be used effectively.

The hybridization step may, for example, be carried out in a solution containing a chaotropic agent such as 50% formamide, a hybrid stabilizing agent such as five times concentrated SSC solution (1x = 0.15M sodium chloride and 0.015M sodium citrate), a buffer such as 0.1M sodium phosphate (pH 7.4), about 100 micrograms (ug)/milliliter (ml) low molecular weight DNA to diminish non-specific binding, 0.1% Triton X-100 to facilitate probe entry into the cells and about 10-20 mM vanadyl ribonucleoside complexes.

All percentages for liquids are on a v/v basis unless otherwise noted.

To the hybridization solution is added a probe population, to hybridize with the target nucleic acids. If the cells are to be ultimately viewed on glass slides (or other solid supports), the cells as either single cell suspensions or as tissue slices are deposited on the slides. The cells are fixed by choosing a fixative which provides the best spatial resolution of the cells and the optimal hybridization efficiency. After fixation, the support bound cells may be dehydrated and stored at room temperature or the hybridization procedure may be carried out immediately.

The hybridization solution containing the probe is added in an amount sufficient to cover the cells. The cells are then incubated at an appropriate temperature.

Temperatures used in the Examples below will be seen to be in the range 42°-46°C. Conditions where preferred temperatures are in the range 50°-55°C have been disclosed in PCT applications WO 90/02173 and WO 90/02204. However, temperatures ranging from 15°C. to 80°C. may be used.

The hybridization solution may include a chaotropic denaturing agent, a buffer, a pore forming agent, a hybrid stabilizing agent, and the target-specific probe molecule.

The chaotropic denaturing agents (Robinson, D. W. and Grant, M. E. (1966) J. Biol. Chem. 241: 4030; Hamaguchi, K. and Geiduscheck, E. P. (1962) J. Am. Chem. Soc. 84: 1329) include formamide, urea, thiocyanate, guanidine, trichloroacetate, tetramethylamine, perchlorate, and sodium iodide. Any buffer which maintains pH at least between 7.0 and 8.0 is preferred.

The pore forming agent is for instance, a detergent such as Brij 35, Brij 58, sodium dodecyl sulfate, CHAPS™ Triton X-100. Depending on the location of the target biopolymer, the pore-forming agent is chosen to facilitate probe entry through plasma, or nuclear membranes or cellular compartmental structures. For instance, 0.05% Brij 35 or 0.1% Triton X-100 will permit probe entry through the plasma membrane but not the nuclear membrane. Alternatively, sodium desoxycholate will allow probes to traverse the nuclear membrane. Thus, in order to restrict hybridization to the cytoplasmic biopolymer targets, nuclear membrane pore-forming agents are avoided. Such selective subcellular localization contributes to the specificity and sensitivity of the assay by eliminating probe hybridization to complementary nuclear sequences when the target biopolymer is located in the cytoplasm. Agents other than detergents such as fixatives may serve this function. Furthermore, a biopolymer probe may also be selected such that its size is sufficiently small to traverse the plasma membrane of a cell but is too large to pass through the nuclear membrane.

Hybrid stabilizing agents such as salts of mono- and di-valent cations are included in the hybridization solution to promote formation of hydrogen bonds between complementary nucleotide sequences of the probe and its target biopolymer. Preferably sodium chloride at a concentration from .15M to 1M is used. In order to prevent non-specific binding of nucleic acid probes, nucleic acids unrelated to the target biopolymers are added to the hybridization solution at a concentration of about 100 fold the concentration of the probe. Specimens are removed after each of the above steps and analyzed by observation of cellular morphology as compared to fresh, untreated cells using a phase contrast microscope. The condition determined to maintain

the cellular morphology and the spatial resolution of the various subcellular structures as close as possible to the fresh untreated cells is chosen as optimal for each step.

Prior to nucleic acid hybridization, the cells may be reacted with antibodies in phosphate buffered saline. After hybridization one may analyze the cells for both bound antibodies and bound hybridization probes.

Mounting Biological Entities/Tissues

Many types of solid supports may be utilized to practice the invention. Supports which may be utilized include, but are not limited to, glass, Scotch tape (3M), nylon, Gene Screen Plus (New England Nuclear) and nitrocellulose. Most preferably glass microscope slides are used. The use of these supports and the procedures for depositing specimens thereon will be obvious to those of skill in the art. The choice of support material will depend upon the procedure for visualization of cells and the quantitation procedure used. Some filter materials are not uniformly thick and, thus, shrinking and swelling during in situ hybridization procedures is not uniform. In addition, some supports which autofluoresce will interfere with the determination of low level fluorescence. Glass microscope slides are most preferable as a solid support since they have high signal-to-noise ratios and can be treated to better retain tissue.

Fixation of Biological Entities/Tissues

A fixative may be selected from the group consisting of any precipitating agent or cross-linking agent used alone or in combination, and may be aqueous or non-aqueous. The fixative may be selected from the group consisting of formaldehyde solutions, alcohols, salt solutions, mercuric chloride sodium chloride, sodium sulfate, potassium dichromate, potassium phosphate, ammonium bromide, calcium chloride, sodium acetate, lithium chloride, cesium acetate, calcium or magnesium acetate, potassium nitrate, potassium dichromate, sodium chromate, potassium iodide, sodium iodate, sodium thiosulfate, picric acid, acetic acid, paraformaldehyde, sodium hydroxide, acetones, chloroform, glycerin, thymol, etc. Preferably, the fixative will comprise an agent which fixes the cellular constituents through a precipitating action and has the following

characteristics: the effect is reversible, the cellular (or viral) morphology is maintained, the antigenicity of desired cellular constituents is maintained, the nucleic acids are retained in the appropriate location in the cell, the nucleic acids are not modified in such a way that they become unable to form double or triple stranded hybrids, and cellular constituents are not affected in such a way so as to inhibit the process of nucleic acid hybridization to all resident target sequences. Choice of fixatives and fixation procedures can affect cellular constituents and cellular morphology; such effects can be tissue specific. Preferably, fixatives for use in the invention are selected from the group consisting of ethanol, ethanol-acetic acid, methanol, and methanol-acetone which fixatives afford the highest hybridization efficiency with good preservation of cellular morphology.

Fixatives for practicing the present invention include 95% ethanol/5% acetic acid for HL-60 and normal bone marrow cells, 75% ethanol/20% acetic acid for K562 and normal peripheral blood cells, 50% methanol/50% acetone for fibroblast cells and normal bone marrow cells, and 10% formaldehyde/90% methanol for cardiac muscle tissue. These fixatives provide good preservation of cellular morphology and preservation and accessibility of antigens, and high hybridization efficiency.

Simultaneously, the fixative may contain a compound which fixes the cellular components by cross-linking these materials together, for example, glutaraldehyde or formaldehyde. While this cross-linking agent must meet all of the requirements above for the precipitating agent, it is generally more "sticky" and causes the cells and membrane components to be secured or sealed, thus, maintaining the characteristics described above. The cross linking agents when used are preferably less than 10% (v/v).

Cross-linking agents, while preserving ultrastructure, often reduce hybridization efficiency; they form networks trapping nucleic acids and antigens and rendering them inaccessible to probes and antibodies. Some also covalently modify nucleic acids preventing later hybrid formation.

Storage of Biological Entities/Tissues

After fixation, microscope slides containing cells may be stored air dried at room temperature for up to three weeks, in cold (4°C) 70% ethanol in water for 6-12 months, or in paraplast for up to two years. If specimens are handled under RNase free conditions, they can be dehydrated in graded alcohols and stored for at least 5 months at room temperature.

Reagents can be purchased from any of a variety of sources including Aldrich Chemical Co., Milwaukee, Wisconsin, Sigma Chemical Co., St. Louis, Missouri, Molecular Probes, Inc., Eugene, Oregon, Clontech, Palo Alto, California, Kodak, Rochester, NY, and SPectrum Chemical Manufacturing Corp., Gardenea, California.

Detection of Oncogenes in Peripheral Blood Cells and Bone Marrow Cells.

In a typical procedure, 10 ml. of human peripheral blood or 2 ml. of human bone marrow cells are incubated at 37° C. in a 1.2% (215 mOs) ammonium oxalate solution to lyse the red blood cells. The white blood cells are centrifuged at 3,000 rpm for 10 minutes in a clinical centrifuge. The cell pellet is subsequently washed with 10 ml. PBS and the pellet is resuspended in PBS. Cells are deposited by cytocentrifugation onto precleaned glass slides and air dried for 5 min. The cells are then fixed in 75% ethanol/ 20% acetic acid for 20 min. at room temperature. Hybridization procedures using oncogene-specific probes are then followed.

Hybridization in solid tissue.

In a typical procedure, four micron thick frozen sections of human breast tissue obtained from surgically removed biopsy samples are mounted on precleaned glass slides and fixed with 50% methanol/50% acetone for 20 min. at room temperature. Hybridization then proceeds using procedures described elsewhere in this document.

One-step in situ hybridization assay

Briefly, cells, either as single cell suspensions or as tissue slices may be deposited on solid supports such as glass slides. Alternatively, cells are placed into a single cell

suspension of about 10^5-10^6 cells per ml. The cells are fixed by choosing a fixative which provides the best spatial resolution of the cells and the optimal hybridization efficiency.

The hybridization is then carried out in the same solution which effects fixation. This solution contains both a fixative and a chaotropic agent such as formamide. Also included in this solution is a hybrid stabilizing agent such as concentrated lithium chloride or ammonium acetate solution, a buffer, low molecular weight DNA and/or ribosomal RNA (sized to 50 bases) to diminish non-specific binding, and a pore forming agent to facilitate probe entry into the cells. Nuclease inhibitors such as vanadyl ribonucleoside complexes may also be included. To the hybridization solution is added a probe (or probes), to hybridize with a target polynucleotide.

The one-step procedure is a means of carrying out the fixation, prehybridization, hybridization and detection steps normally associated with in situ hybridization procedures all in one step. By modifying the components of this "one-step" solution, a convenient temperature may be used to carry out the hybridization reaction. Furthermore, this provides a hybridization assay which can be accomplished with viable or non-viable cells in solution. In either case, the assay is rapid and sensitive.

Treatment of Sample for the one-step procedure

Regardless of whether the cell specimen is in suspension or on solid supports, the hybridization procedure is carried out utilizing a single hybridization solution which also fixes the cells. This fixation is accomplished in the same solution and along with the hybridization reaction. The fixative may be selected from the group consisting of any precipitating agent or cross-linking agent used alone or in combination, and may be aqueous or non-aqueous.

Cells Preparation in the "one-step" procedure

Tissue samples are broken apart by physical, chemical or enzymatic means into single cell suspension. Cells are placed into a PBS solution (maintained to cellular osmolality with bovine serum albumin (BSA) at a concentration of 10^5 to 10^6 cells per ml. Cells in suspension may be fixed and processed at a later time, fixed and processed immediately,

or not fixed and processed in the in situ hybridization system of the present invention.

A single solution is added to the cells/tissues (hereafter referred to as the specimen). This solution contains the following: a mild fixative, a chaotrope, a nucleic acid probe (RNA or DNA probe which is prelabeled) and/or antibody probe, salts, detergents, buffers, and blocking agents. The incubation in this solution can be carried out at 55°C for 20 minutes as well as other conditions such as those in the Examples below.

The fixative is one which has been found to be optimal for the particular cell type being assayed (eg., there is one optimal fixative for bone marrow and peripheral blood even though this "tissue" contains numerous distinct cell types). The fixative is usually a combination of precipitating fixatives (such as alcohols) and cross-linking fixatives (such as aldehydes), with the concentration of the cross-linking fixatives kept very low (less than 10%). Frequently, the solution contains 10-40% ethanol, and 5% formalin. The concentration and type of precipitating agent and crosslinking agent may be varied depending upon the probe and the stringency requirements of the probe, as well as the desired temperature of hybridization. Typical useful precipitating and cross-linking agents are specified in PCT applications WO 90/02173 and WO 90/02204.

The hybridization cocktail contains a denaturing agent, usually formamide at about 30% (v/v), but other chaotropic agents such as NaI, urea, etc. may also be used. Furthermore, several precipitating and/or cross-linking fixatives also have mild denaturing properties; these properties can be used in conjunction with the primary denaturant in either an additive or synergistic fashion. The hybridization cocktail may be constructed to preferentially allow only the formation of RNA-RNA or RNA-DNA hybrids. This is accomplished by adjusting the concentration of the denaturing agents along with the concentration of salts (primarily monovalent cations of the Group I series of metals along with the ammonium ion) and along with the temperature of hybridization which is used. This allows for the selective hybridization of probe to either cellular RNA or DNA or both RNA and DNA simultaneously with distinct probes. This further allows the probes to be supplied in a premixed solution which presents the optimal conditions for generating a signal and minimizing noise while simultaneously optimally "fixes" the morphology of the cells/tissues.

The present invention may be provided in the form of a kit adapted for a one-step process. Therefore, another invention is a kit for detecting a nucleic acid molecule in a biological entity, said kit comprising a probe population described herein and one more reagents for use in a solution for reacting said probe population with said biological entity so that a hybrid molecule can form between a molecule of the probe population and a nucleic acid molecule in the biological entity. In another aspect, one of the present inventions is a kit of wherein the biological entity is a cell and the one or more reagents comprise a reagent selected from the group, a fixative and a chaotropic agent. (Preferred are the fixatives and chaotropic reagents identified in this application.)

For example, a kit could include a solution containing a fixation/hybridization cocktail and one or more labeled probes. This solution could, for example, contain 15-40% ethanol, 25-40% formamide, 0-10% formaldehyde, 0.1-1.5 M LiC1, 0.05-0.5 M Tris-acetate (pH 7-8), 0.05%-0.15% Triton X-100, 20 ug/ml-200 ug/ml of a non-specific nucleic acid which does not react with the probe(s), and 0.1 ug/ml to 10 ug/ml of single stranded probes

directly labeled with a reporter molecule. More specifically, for example, this solution could contain 30% ethanol, 30% formamide, 5% formaldehyde, 0.8M LiC1, 0.1M Tris-acetate (pH 7.4), 0.1% Triton X-100, 50 ug/ml of the non-specific nucleic acid, and 2.5 ug/ml of each single stranded probes directly labeled with a fluorescent reporter molecule.

Additionally, there would preferably be means and instructions for performing the said in situ hybridization reaction of the present invention.

Additionally, a kit may also include:

1. A second detectable reporter system which would react with the probe or the probe-target hybrid.

2. Concentrated stock solution(s) to be diluted sufficiently to form wash solution(s).

3. Any mechanical components which may be necessary or useful to practice the present invention such as a solid support (e.g. a microscope slide), an apparatus to affix cells to said support, or a device to assist with any incubations or washings of the specimens.

4. A photographic film or emulsion with which to record results of assays carried out with the present invention.

Examples

Example 1

Demonstration that 25-base oligomers hybridize while 6- to 12-base oligomers do not under the hybridization conditions of the Example

Preparation of Cells

The H9 cell line was used in the following experiment. Cultured cells were washed with nuclease-free Phosphate Buffered Saline (PBS) and placed in a single cell suspension at a concentration that resulted in clearly separated cells. The cells were spun down to a pellet and the supernatant. drained off. The cells were resuspend in 40% ethanol, 50% PBS, and 10% glacial acetic acid and left for 12-16 hours at 4°C. After fixation, the cells were spun to remove the fixative and then washed once in 1X PBS and resuspend in 2X SSC. The cells should be used immediately.

Preparation Of Probes

For a positive control probe, a conserved segment of the eukaryotic 28S rRNA was designed and utilized; it was designated 28S-25-AL (SEQ ID NO:1) and it served as a positive probe for the experiment described herein. The negative probe, designated NR-25-AL (SEQ ID NO:9), was derived from the nitrogen reductase gene found in bacteria and was known to not hybridize to nucleic acid within eukaryotic cells. The DNA sequences for these two probes used are shown in Table 1 below. Twelve base, ten base, eight base, and six base oligomers, derived from these 25-base oligomers were also

prepared with the sequences shown in the Table 1 below. All sequences displayed in the Examples have the 5' end as the left end of the sequence.

Table 1

Probe Designation	Sequence
28S-25-AL	ATCAGAGTAGTGGTATTTCACCGGC (SEQ ID NO:1)
28S-21-AL	ATCAGAGTAGTGGTATTTCAC (SEQ ID NO:2)
28S-18-AL	ATCAGAGTAGTGGTATTT (SEQ ID NO:3)
28S-15-AL	ATCAGAGTAGTGGTA (SEQ ID NO:4)
28S-12-AL	ATCAGAGTAGTG (SEQ ID NO:5)
28S-10-AL	ATCAGAGTAG (SEQ ID NO:6)
28S-8-AL	ATCAGAGT (SEQ ID NO:7)
28S-6-AL	ATCAGA (SEQ ID NO:8)
NR 25-AL	TACGCTCGATCCAGCTATCAGCCGT (SEQ ID NO:9)
NR 12-AL	TACGCTCGATCC (SEQ ID NO:10)
NR 10-AL	TACGCTCGAT (SEQ ID NO:11)
NR 8-AL	TACGCTCG (SEQ ID NO:12)
NR 6-AL	TACGCT (SEQ ID NO:13)

Probe Synthesis, & Labelling

The oligodeoxynucleotides were synthesized (Applied Biosystems DNA Synthesizer Model 380 B using the recommended A.B.I. reagents), and in the last stage an aminohexyl linker was attached to the 5' end phosphate. The 5'-aminohexyl

oligodeoxynucleotides were were purified and coupled to a rhodamine derivative from Molecular Probes and purified by Waters HPLC using a baseline 810 chromatography work station.

Hybridization

For the hybridization procedure, to pelleted cells was added 50 μl of an hybridization cocktail consisting of 30% formamide, 5X SSC, O.16M sodium phosphate buffer, pH 7.4, 1 μg/μl sheared DNA, 3% (v/v) Triton X-100 (alcohol derivative of polyoxylene ether, see Aldrich Chemical Co. catalogue for 1990-91), 5% PEG 4000 (polyethylene glycol), 25 mM DTT (dithiothreitol), 0.4 M guanidinium isothiocyanate, 15X Ficoll/PVP, and the probe added at a concentration of 2.5 μg/ml. Hybridizations were carried out at 42°C for 30 minutes. In the foregoing, 500X Ficoll/PVP is 5g of Ficoll type 400 (polysucrose 400,000 mol wt) plus 5 g of PVP (polyvinylpyrollidone) dissolved in water to a total volume of 100 ml; 15X FIcoll/PVP is 500X Ficoll/PVP diluted with water by a factor of 15/500.

Washing

Proper washing after the hybridization reaction is essential to eliminate background due to non-specific binding of probe. Post-hybridization the cells were placed in a 15 ml conical tube to which was added 10 ml of a wash solution preheated to 42°C, consisting of 0.1X SSC, 0.4M guanidinium isothiocyanate, and 0.1% Triton. The solution was agitated until the cells were a single cell suspension and then spun at 250 X g for 5 minutes. The supernatant was removed and to the pellet was added 10 ml of a wash solution preheated to 42°C, consisting of 0.1X SSC, 0.1% Triton. The solution was agitated until the cells were a single cell suspension. The cells were spun at 250 X g for

5 minutes. The supernatant was removed and the cell pellet resuspended in 0.2 ml counterstain solution consisting of 0.0025% Evans Blue in 1X PBS.

Flow Cytometer Use and Interpretation

The cells were analyzed on a Profile II™ made by Coulter Instruments. The Instrument uses a 488nm argon laser, a 525nm band pass filter for FL1 and a 635nm band pass filter for the counterstain. For each sample analyzed the sample containing the negative probe was analyzed first and the quad-stats were set so that less than 0.01% of the cells fell in the upper-right quadrant. Next the sample analyzed with the positive probe was analyzed under the exact same parameters as the sample analyzed with the negative probe. Since the quad-stats were set correctly and the two samples had been handled identically, any number of cells (above 0.01%) that were recorded in the upper right quadrant were scored as positive.

Sequence of steps

Approximately 500,000 H9 cells were equally divided into two tubes and fixed as described above. For one of these sample aliquots was added a hybridization solution containing a positive probe (28S) and to the other a negative probe (NR), corresponding to the same size as the positive probe as in the list in Table 1 above. Following hybridization and washing, flow cytometry was performed.

Results

Using flow cytometry, the following results were obtained: >99% of the cells were positive when probe 28S-25-AL (SEQ ID NO:1) was used, between 0.01% and 99% of the cells were positive when the 28S-21-AL (SEQ ID NO:2) probe was used, and <.01% were

positive when any of the other probes were used. Furthermore if the mean LFL1 was measured, the results were as in Table 2.

Table 2

	Signal (Mean LFL1)		
Length	NR	28S	Fold Diff.
6	.322	.370	1.1
8	6.4	.441	Neg
10	.422	.3	.70
12	.321	.231	.72
15	.327	.373	1.1
18	.407	.339	.83
21	.281	.616	2.2
25	.3	50.0	168

Example 2

Demonstration that 25-base oligomers made to the "overlap" opposite strand of DNA increases the intensity of the signal in a linear fashion

Preparation of Cells

A cell line with an additional chromosome 18 (XX=18) was grown as a monolayer to confluency and then trypsinized and approximately 5,000 cell were deposited onto a clean glass slide by the cytospining method.

Preparation Of Probes

The sequences for the 25-base synthetic oligonucleotide probes listed below in Table 3 and in Figure 1 were obtained from the published sequences for the alpha centromeric repetitive DNA sequence on chromosome 18.

In Figure 1, the nucleotide sequence in the top strand starting at position 100 and ending at position 212 is SEQ ID NO:25 for a segment of double-stranded DNA.

In Figure 1, the sequence in the bottom strand starting at position 225 and ending at position 101 is SEQ ID NO:26.

In Figure 1, the nucleotide sequence in the top strand starting at position 288 and ending at position 403 is SEQ ID NO:27 for a segment of double-stranded DNA.

In Figure 1, the sequence in the bottom strand starting at position 287 and ending at position 276 is SEQ ID NO:28.

In Figure 1, the sequences H18-10, H18-11, H18-100L, H18-100R, and H18-110R are SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, and SEQ ID NO:18, respectively.

Table 3

Probe Designation	Sequence
H18-10	ACTCTACACACATGAGTGTGATTCT (SEQ ID NO:14)
H18-11	CTTAACTTGGTGGCAAAACTTCCTC (SEQ ID NO:15)
H18-10-0L	CTCAGAACTTATTTGAGATGTGTGT (SEQ ID NO:16)
H18-10-OR	ACTCACACTAAGAGAATTGTTCCAC (SEQ ID NO:17)
H18-11-OR	CGTTTTGAAGGAGCAGTTTTGAAAC (SEQ ID NO:18)

Probe Synthesis, & Labelling

The oligodeoxynucleotides were synthesized (Applied Biosystems DNA Synthesizer Model 380 B using the recommended A.B.I. reagents), and in the last stage an aminohexyl

linker was attached to the 5' end phosphate. The 5'-aminohexyl oligodeoxynucleotides were then coupled to a rhodamine dye from Clontech and purified by Waters HPLC using a baseline 810 chromatography work station.

Hybridization

For the hybridization procedure, to the cells deposited onto the slides was added 20 to 25 μl of a hybridization cocktail consisting of 30% formamide, 5X SSC, O.1 M sodium phosphate buffer, pH 7.4, 100 μg/ml low molecular weight, denatured, salmon or herring sperm DNA, 5% (v/v) Triton X-100, 15X Ficoll/ PVP, 0.4 M guanidinium isothiocaynate, 10 mM DTT, and 0.025 M EDTA and the probe, added at a concentration of 2.5 μg/ml. Denaturation and hybridization was carried out simultaneously by placing the slides in an incubator for 15 minutes at 85°C.

Washing

Proper washing after the hybridization reaction is essential to eliminate background due to non-specific binding of probe. Post-hybridization, the slides were placed in a coplin jar to which was added 100 ml of a wash solution, consisting of 0.1X SSC and 0.4 M guanidinium isothiocyanate and 0.1% Triton X-100. The solution was agitated and held in this solution for 2 minutes. This wash solution was removed and a second wash solution, consisting of 0.1X SSC and 0.1% Triton X-100 was added. This solution was agitated for 5 minutes and poured off. Then 15 ul. of mounting solution, containing 0.1% 1,4 phenylenediamine antifade in 50% glycerol and 1 ug/ml Hoechst (33258) was used.

Fluorescence Detection

Photomicrographs were taken on an Olympus BH10 microscope with fluorescence capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 20-second exposure time was consistently used, so that direct comparisons could be made between all photomicrographs taken.

Results

Visual inspection of slides revealed that, the signal intensity obtained with all probes was about twice that obtained with the probes for the sense strand alone or the anti-sense strand alone.

Example 3

This example demonstrated that 25-base oligomers can efficiently hybridize to one strand of DNA while a second 25-mer will hybridize to the opposite strand. The probes were designed such that the probes made to detect one strand of the DNA overlapped twelve to thirteen bases of the probes designed to detect the other strand of DNA. See Fig. 2 for probe structures and how they hybridize to the their targets.

In Figure 2, the SEQ ID numbers for the designated segments are summarized in Table 7.

Table 7

Segment number	SEQ ID NO:
501	30
502	31
503	32
504	33
505	34
506	35
507	36
508	37
509	38
510	39
511	40
512	41
426	44
427	45
428	46
429	47
430	48
431	49
432	50
433	51
434	52
435	53
436	54

In Figure 2, in the top strand, the first eight bases starting from the left are SEQ ID NO:29.

In Figure 2, in the bottom strand, the 20-base sequence to the left of segment 426 is SEQ ID NO:43.

In Figure 2, in the top strand, the 9-base sequence at the end of the strand is SEQ ID NO:42.

In Figure 2, in the bottom strand, the 22-base sequence that starts from the right end in the last line of the figure and ends just before segment 436 is SEQ ID NO:55.

Preparation of Cells

Two cell lines were used for this example.

1. HTB 31 "C-33A" is a human cervical carcinoma derived cell line from cervical cancer biopsies (J. National Cancer Institute 32:135-148, 1964) and contains no human papilloma virus was used as the negative control.
Culture Media: Eagles MEM with non-essential Amino Acids, sodium pyruvate, 10% fetal bovine serum.

2. CCL 1550 "CAski" is a human cervical carcinoma cell line containing 400-500 copies of HPV16 integrated into its genome.(Science 196:1456-1458, 1977), and was used as the positive control.
Culture Media: RPM I 1640 with L-glutamine, and 10% fetal bovine serum.

Cells from both cell lines were grown to confluence in 5% CO2, in 100 ml culture flasks. They were rinsed 1 time in 1X PBS. To the cells was added 2 ml of 0.25% Trypsin, in 0.02 EDTA. These were incubated for 5 minutes at 37 °C, gently tapped to dislodge cells. To these cells were add 10 ml. of their respective media. 5 x 10^3 cells were then cytospun for 7 minutes at 700 rpm's onto clean glass slides, and left to air dry. To these cells was added 20 ul of ethanol:methanol (3:1). They were then allowed to air dry.

Preparation Of Probes

The 25-base synthetic oligonucleotide probes listed in Figure 1 below and designated HPV 16-426-436 and HPV 16-501-512, was obtained from the published sequence for HPV type

16 and was accessed via the Genetic Sequence Data Bank, GenBank, version 69.0.

Probe Synthesis, & Labelling

The oligodeoxynucleotides were synthesized (Applied Biosystems DNA Synthesizer Model 380 B using the recommended A.B.I. reagents), and in the last stage an aminohexyl linker was attached to the 5' end phosphate. The 5'-aminohexyl oligodeoxynucleotides were then coupled to a rhodamine dye from Clontech and purified by Waters HPLC using a baseline 810 chromatography work station.

Hybridization

For the hybridization procedure, 20 μl of an hybridization cocktail consisting of PEG 21%, formamide 25%, 5X SSC, salmon sperm DNA 1 mg/ml, Ficoll/PVP 15X, 0.4 M guanidinium isothiocyanate, 50 mM DTT, 5% Triton X-100, 50 mM EDTA, 50 mM Na_2PO_4, and probe at a concentration of 0.06 ug/ul is added to the slide. A coverslip was applied and the slide was heated to 95°C for 5 minutes, allowed to cool to 42°C and incubated for 25 minutes at that temperature.

Washing

Post-hybridization, the slides were placed in a coplin jar to which was added 100 ml of a wash solution, consisting of 0.1X SSC and 0.4M guanidinium isothiocyanate and .1% Triton X100. The solution was agitated and held in this solution for 2 minutes. This wash solution was removed and a second wash solution, consisting of 0.1X SSC and 0.1% Triton X100 was added. This solution was agitated for 1 minutes and poured off and this last wash was repeated 2 times. Following the washes, 8 ul. of Antifade / Hoechst counterstain was added. The slides were coverslipped, and viewed under the fluorescent microscope.

Fluorescence Detection

Photomicrographs were taken on an Olympus BH10 microscope with fluorescence

capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 20-second exposure time was consistently used, so that direct comparisons could be made between all photomicrographs taken.

The cell lines C-33A and Caski were used to determine the intensity difference between the signal obtained using probes directed at one strand of the DNA vs probes directed at both strands ("staggered overlap" probes).

The results in Table 4 show that when 12 oligos of either sense strand or anti-sense strand probe were used, the intensity of the signal was one-half the intensity of that obtained when both strands were used in the hybridization solution.

Table 4

Cells	Probe	Label	#Oligos	Results (Intensity)
C-33A	Sense	FITC	12	-
Caski	Sense	FITC	12	++
C-33A	Antisense	FITC	12	-
Caski	Antisense	FITC	12	++
C-33A	Sense & Antisense	FITC	24	-
Caski	Sense & Antisense	FITC	24	++++

Example 4

Demonstration that 25-base oligomers hybridize while 6-12-base oligomers do not, under the hybridization conditions used

Preparation of Cells

Approximately 5000 white blood cells from a normal male human donor were deposited onto slides by the cytospin method.

Preparation Of Probes

The sequences for the 25-base synthetic oligonucleotide probes listed below and designated HYR 7 were obtained from the published sequences for the alpha centromeric repetitive DNA sequence on the Y chromosome. Twelve base, ten base, eight base, and six base oligomers, derived from these 25-base oligomers were also prepared as shown in the Table 5 below.

Table 5

Probe Designation	Sequence
HYR-7-25	GAGTCGATTTTATTGCATTAGATTC (SEQ ID NO:19)
HYR-7-15	GAGTCGATTTTATTG (SEQ ID NO:20)
HYR-7-12	GAGTCGATTTTA (SEQ ID NO:21)
HYR-7-10	GAGTCGATTT (SEQ ID NO:22)
HYR-7-8	GAGTCGAT (SEQ ID NO:23)
HYR-7-6	GAGTCG (SEQ ID NO:24)

Probe Synthesis, & Labelling

The oligodeoxynucleotides were synthesized (Applied Biosystems DNA Synthesizer Model 380 B using the recommended A.B.I. reagents), and in the last stage an aminohexyl linker was attached to the 5' end phosphate. The 5'-aminohexyl oligodeoxynucleotides were then coupled to a rhodamine dye from Clontech and purified by Waters HPLC using a baseline 810 chromatography work station.

Hybridization

For the hybridization procedure, to the cells deposited onto the slides was added 20 to 25 μl of a hybridization cocktail consisting of 30% formamide, 5X SSC, O.1M sodium phosphate buffer, pH 7.4, 100 μg/ml low molecular weight, denatured, salmon or herring sperm DNA, 5% (v/v) Triton X-100, 15X Ficoll/ PVP, 0.4 M guanidinium isothiocaynate, 10 mM DTT, and 0.025 M EDTA and the probe, added at a concentration of 2.5 μg/ml. Denaturation and hybridization was carried out simultaneously by placing the slides in an incubator for 15 minutes at 85°C.

Washing

Proper washing after the hybridization reaction is essential to eliminate background due to non-specific binding of probe. Post-hybridization, the slides were placed in a coplin jar to which was added 100 ml of a wash solution, consisting of 0.1X SSC and 0.4M guanidinium isothiocyanate and 0.1% Triton X-100. The solution was agitated and held in this solution for 2 minutes. This wash solution was removed and a second wash solution, consisting of .1X SSC and 0.1% Triton X-100 was added. This solution was agitated for 5 minutes and poured off. Then 15 ul. of mounting solution, containing 0.1% 1,4 phenylenediamine antifade in 50% glycerol and 1 ug/ml Hoechst (33258) was used.

Fluorescence Detection

Photomicrographs were taken on an Olympus BH10 microscope with fluorescence

capabilities, using Kodak Ektachrome EES-135 (PS 800/1600) film, exposed, and push processed at 1600 ASA. A 20-second exposure time was consistently used, so that direct comparisons could be made between all photomicrographs taken.

Results

The results showed that detectable hybridization was found with the HYR-7-15 (SEQ ID NO:20) and HYR-7-25 (SEQ ID NO:19) probes but not with either of the other probes, HYR-7-12 (SEQ ID NO:21), HYR-7-10 (SEQ ID NO:22), HYR-7-8 (SEQ ID NO:23), and HYR-7-6 (SEQ ID NO:24) (See, for example, Figs. 3a and 3b).

Example 5

Demonstration that 25-base oligomers hybridize while 6-12-base oligomers do not, under the hybridization conditions used.

Preparation of Cells and DNA and Southern Blot

The cell line (GM 02504G, Coriell Inst. of Med. Research, Camden NJ), grown as a monolayer and were trypinsized. DNA isolated essentially by the method of Maniatis et al (Molecular Cloning, T. Maniatis, E.F. Fritsch and J. Sambrook, eds., Cold Spring Harbor Laboratory, NY, 1982) and digested to completion using restriction enzymes Bam H1 and EcoR1 under conditions described by Maniatiset al. Then an aliquot of 10 ug of each digested DNA was electrophoresed from a 2 mm-wide slot through a 1.25 percent agarose gel. The electrophoretically fractionated DNA was then immobilized on nitrocellulose filter paper using the procedure of Southern (see Maniatis et al).

Preparation of Probes

The sequences for the 25-base synthetic oligonucleotide probes listed below and designated HYR 7 were obtained from the published sequences for the alpha centromeric repetitive DNA sequence on the Y chromosome. Twelve base, ten base, eight base, and six base oligomers, derived from these 25-base oligomers were also prepared as shown in the Table 6 below.

Table 6

Probe Designation	Sequence
HYR-7-25	GAGTCGATTTTATTGCATTAGATTC (SEQ ID NO:19)
HYR-7-12	GAGTCGATTTTA (SEQ ID NO:21)
HYR-7-10	GAGTCGATTT (SEQ ID NO:22)
HYR-7-8	GAGTCGAT (SEQ ID NO:23)
HYR-7-6	GAGTCG (SEQ ID NO: 24)

Probe Synthesis & Labeling

The above oligodeoxynucleotides were synthesized using an Applied Biosystems DNA Synthesizer Model 380 B and using the recommended A.B.I. reagents and purified by waters

HPLC using a baseline 810 chromatography work station.

The probes were then end labeled with digoxigenin at the 3' end using an end labeling kit from Boehringer Mannheim Biochemicals (BMB) and using the BMB recommended procedure.

Hybridization

The filters were cut to a size of about 10 cm x 2 cm and were incubated for 3 hrs at 65° C in a pre-hybridization solution followed by incubation at 56° C overnight in a hybridization solution containing end-labeled oligonucleotide probes.

The hybridization cocktail consisted of 30% formamide, 5X SSC, 0.1M sodium phosphate buffer, pH 7.4, 100 ug/ml low molecular weight denatured salmon or herring sperm DNA, 5% (v/v) Triton X-100, 15X Ficoll/PVP, 0.4 M guanidinium isothiocyanate, 10 mM DTT, and 0.025 M EDTA and the probe, added at a concentration of 2.4 ug/ml (micrograms/ml).

Washing

After hybridization, the filters were washed, blocked, equilibrated and reacted with anti-anti-digoxigenin/alkaline phosphatase conjugate according to BMB protocol and soaked in the substrate (lumipos 530, BMB). The filters were then exposed to x-ray film and the films were developed.

Results

The results showed that detectable hybridization was found with the HYR-7-25 (SEQ ID NO:19) probe but not with either of the other probes, HYR-7-12 (SEQ ID NO:21), HYR-7-10 (SEQ ID NO:22), HYR-7-8 (SEQ ID NO:23), and HYR-7-6 (SEQ ID NO:24).

Example 6

The Use Of Synthetic Oligonucleotides As Probes For Both Strands Of DNA As Targets For Hybridization

Example 6

This Example demonstrates that oligomers prepared to both strands of a DNA target and that the results can be monitored by flow cytometry. It also demonstrates the ability to hybridize to both DNA strands allows one to quantitate simultaneously the amount of DNA and RNA within individual cells.

Preparation of Cells

The H9 cell line is used in the following experiment. Cultured cells are washed with nuclease-free Phosphate Buffered Saline (PBS) and placed in a single cell suspension at a concentration that results in clearly separated cells. The cells are spun down to a pellet and the supernatent drained off. The cells are resuspended in 40% ethanol, 50% PBS, and 10% glacial acetic acid and left for 12-16 hours at 4°C. After fixation, the cells are spun to remove the fixative and then washed once in 1X PBS and resuspended in 2X SSC. The cells should be used immediately.

Preparation of Probes

The HIV sequences used as probes are accessed via GenBank, version 69.0, prepared as probe by cutting them into 30-mers as described in figure 2, for HPV sequences. This design results in an "overlap" region of 15 bases.

Probe Designation	GenBank Locus Name	Fluorescent Label	Molecular Probes, Inc. Cat. #
HIV - sense strand	HUMHB102	FITC	I-3
HIV - antisense strand	HUMHB102	rhodamine derivative	T488

Probe Synthesis & Labeling

The above mentioned sequences are cut into 30-base oligonucleotides and synthesized as phosphorothioate oligonucleotides using DNA synthesizers (Applied Biosystem DNA Synthesizer, Model 380B) and using the recommended ABI reagents. The polysulfurized oligonucleotides are then coupled to a fluorescent dye and purified by column chromatography and HPLC. 30-base NR oligonucleotides (30-mers) serve as the negative control probes.

Probes are made as phosphorothioate oligonucleotides, each 30-mer having four sulfur atoms, using an Applied Biosystem (ABI) DNA Synthesizer, Model 380B and the recommended ABI reagents. The sulfur atoms are located as follows: one is at the extreme 5' end of the probe, a second is between the 7th and 8th nucleosides (counting from the 5' end), the third is between the 22nd and 23rd nucleosides, and the fourth is between the 29th and 30th nucleosides. The sulfur atoms of the polysulfurized oligonucleotides are then coupled to a fluorescent dye, iodoacetamido-fluorescein, as follows (smaller amounts can be synthesized by adjusting the volumes): 200 μg of dried oligonucleotide is dissolved in 100 μl of 250 mM Tris buffer, pH 7.4 to form a first solution. Then one mg of iodoacetamido-fluorescein is combined with 100 μl of dry dimethylformamide (i.e., 100 percent DMF) in

a second solution. The two solutions are mixed together and shaken overnight. After the overnight incubation, the labeled oligonucleotide is precipitated with ethanol and 3M sodium acetate. This crude material is then loaded on to a PD-10 column to remove free dye. The desired fractions are then collected. The liquid phase is then removed under vacuum. The crude material is then purified with HPLC (high performance liquid chromatography).

Hybridization

For the hybridization procedure, to pelleted cells is added 50 μl of a hybridization cocktail consisting of 30% formamide, 5X SSC, 0.16M sodium phosphate buffer, pH 7.4, 1 μg/μl sheared DNA, 3% (v/v) Triton X-100, 5% PEG 4000, 25mM DTT, .4M guanidinium isothiocyanate, 15X Ficol/PVP, and the probe added at a concentration of 2.5 μg/ml. Hybridizations are carried out at 42°C for 30 minutes.

Washing

Proper washing after the hybridization reaction is essential to eliminate background due to non-specific binding of probe. Post-hybridization the cells are placed in a 15 ml conical tube to which is added 10 ml of a wash solution, consisting of .1X SSC, .4M guanidinium isothiocyanate, and .1% Triton at a temperature of 42°C. The solution is agitated until the cells are a single cell suspension and then spun at 250 X g for 5 minutes. The supernatant is removed and to the pellet is added 10 ml of a wash solution, consisting of .1X SSC, .1% Triton at a temperature of 42°C. The solution is agitated until the cells are a single cell suspension. The cells are spun at 250 X g for 5 minutes. The supernatant is removed and the cell pellet resuspended in 0.2 ml counterstain solution consisting of .0025% Evans Blue in 1X PBS.

Flow Cytometer Use and Interpretation

The cells are analyzed on a FACSTAR™ made by Beckon Dickinson. The Instrument

uses a 5 watt argon laser coupled to a dye head, a 525nm band pass filter for FL1 and a 584nm band pass filter for the Rhodamine. For each sample analyzed the sample containing the negative probe is analyzed first and the quad-stats are set so that less than 0.01% of the cells fall in the upper-right quadrant or lower-right quandant. Next the sample analyzed with the HIV probes is analyzed under the exact same parameters as the sample analyzed with the negative probe. Since the quad-stats are set correctly and the two samples have been handled identically, any number of cells (about 0.01%) that are recorded in the upper right quadrant are scored as positive for both strands and/or mRNA. Any number of cells (above 0.01%) that are recorded in the lower right quadrant are scored positive for DNA only.

The Histogram is constructed so that FL-3 is the Y axis and FL-1 is the X axis.

Example 7

Alternative protocols for hybridization

The protocol of Example 4or 6 can be followed with one or more of the following changes:

1) the hybridization cocktail additionally contains 10% DMSO (v/v) and 5% (v/v) vitamin E;

2) Instead of adding 50 ul of hybridization cocktail to the pelleted cells, 45 ul of hybridization cocktail is added to 2.5 ul of squalane and 2.5 ul of pyrrolidinone and the resulting 50 ul is added to the pelleted cells; and

3) 10% (v/v) dodecyl alcohol is added to the solution in which the cells are suspended for flow cytometric analysis,

4) 5 μl of 1 M (1 molar) DTT and 5 μl of Proteinase K (1 mg/ml) solution are added to 100 μl of cocktail and the hybridization reaction is run, for example, at 42°C for 5 min, then at 95°C for 5 min, and then at 42°C for 2 min, and

5) 0.05% or 0.10% aurintricarboxylic acid is added to the hybridization cocktail.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Prashad, Nagindra
Blick, Mark
Weber, William D
Cubbage, Michael L
Ju, Shyh C
Asgari, Morteza

(ii) TITLE OF INVENTION: The Use of Oligonucleotide Probes for Both Strands of a DNA Target

(iii) NUMBER OF SEQUENCES: 55

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(B) STREET: 20 West Third Street
(C) CITY: Media
(D) STATE: PA
(E) COUNTRY: USA
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy Disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/915,900
(B) FILING DATE: 17-JUL-1992
(C) CLASSIFICATION:

(vii) ATTORNEY/AGENT INFORMATION
(A) NAME: Elman, Gerry J
(B) REGISTRATION NUMBER: 24,404
(C) REFERENCE/DOCKET NUMBER: M19-026

(viii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215 892 9580
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(2) INFORMATION FOR SEQ ID NO:1

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCAGAGTAG TGGTATTTCA CCGGC 25

(2) INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATCAGAGTAG TGGTATTTCA C 21

(2) INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATCAGAGTAG TGGTATTT 18

(2) INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGAGTAG TGGTA 15

(2) INFORMATION FOR SEQ ID NO:5

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATCAGAGTAG TG 12

(2) INFORMATION FOR SEQ ID NO:6

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATCAGAGTAG 10

(2) INFORMATION FOR SEQ ID NO:7

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATCAGAGT 8

(2) INFORMATION FOR SEQ ID NO:8

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: cDNA to rRNA

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATCAGA 6

(2) INFORMATION FOR SEQ ID NO:9

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TACGCTCGAT CCAGCTATCA GCCGT 25

(2) INFORMATION FOR SEQ ID NO:10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACGCTCGAT CC 12

(2) INFORMATION FOR SEQ ID NO:11

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TACGCTCGAT 10

(2) INFORMATION FOR SEQ ID NO:12

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TACGCTCG 8

(2) INFORMATION FOR SEQ ID NO:13

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TACGCT 6

(2) INFORMATION FOR SEQ ID NO:14

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

ACTCTACACA CATGAGTGTG ATTCT 25

(2) INFORMATION FOR SEQ ID NO:15

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTTAACTTGG TGGCAAAACT TCCTC 25

(2) INFORMATION FOR SEQ ID NO:16

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTCAGAACTT ATTTGAGATG TGTGT 25

(2) INFORMATION FOR SEQ ID NO:17

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACTCACACTA AGAGAATTGT TCCAC 25

(2) INFORMATION FOR SEQ ID NO:18

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGTTTTGAAG GAGCAGTTTT GAAAC 25

(2) INFORMATION FOR SEQ ID NO:19

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GAGTCGATTT TATTGCATTA GATTC 25

(2) INFORMATION FOR SEQ ID NO:20

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GAGTCGATTT TATTG 15

(2) INFORMATION FOR SEQ ID NO:21

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAGTCGATTT TA 12

(2) INFORMATION FOR SEQ ID NO:22

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GAGTCGATTT 10

(2) INFORMATION FOR SEQ ID NO:23

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GAGTCGAT 8

(2) INFORMATION FOR SEQ ID NO:24

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GAGTCG 6

(2) INFORMATION FOR SEQ ID NO:25

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 112 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

ATAGAGCAGG TTTGAATCAC TCCTTTTGTA GTATCTGGAA 40

GTGGACATTT GGAGGCTTTC AGGCCTATGT TGGAAAAGGA 80

AATATCTTCC ATAACAACTA GACAGAAGCA TT 112

(2) INFORMATION FOR SEQ ID NO:26

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AATAAGTTCT GAG 13

(2) INFORMATION FOR SEQ ID NO:27

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 113 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

ACTCTTTTTC TGGAATCTGC AAAGTGGATA TTTGGCTAGC 40

TTTGGGGATT TCGCTGGAAC GGAATACATA TAAAAAGCAC 80

ACAGCAGCGT TCTGAGAAAC TGCTTTCTGA TGT 113

(2) INFORMATION FOR SEQ ID NO:28

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTTTCAAAAC TG 12

(2) INFORMATION FOR SEQ ID NO:29

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

AACAC AGT 8

(2) INFORMATION FOR SEQ ID NO:30

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AACTAGTAGC ACACCCATAC CAGGG 25

(2) INFORMATION FOR SEQ ID NO:31

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TCTCGCCCAG TGCCACGCCT AGGAT 25

(2) INFORMATION FOR SEQ ID NO:32

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TATATAGTCG CACAACACAA CACGT 25

(2) INFORMATION FOR SEQ ID NO:33

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TAAAGTTGTA GACCCTGCTT TTGTA 25

(2) INFORMATION FOR SEQ ID NO:34

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ACCACTCCCA CTAAACTTAT TACAT 25

(2) INFORMATION FOR SEQ ID NO:35

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

ATGATAATCC TGCATATGAA GGTAT 25

(2) INFORMATION FOR SEQ ID NO:36

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGATGTGGAT AATACATTAT ATTTT 25

(2) INFORMATION FOR SEQ ID NO:37

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TCTAGTAATG ATAATAGTA TAATA 25

(2) INFORMATION FOR SEQ ID NO:38

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TAGCTCCAGA TCCTGACTTT TTGGA 25

(2) INFORMATION FOR SEQ ID NO:39

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TATAGTTGCT TTACATAGGC CAGCA 25

(2) INFORMATION FOR SEQ ID NO:40

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TTAACCTCTA GGCGTACTGG CATTA 25

(2) INFORMATION FOR SEQ ID NO:41

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGTACAGTAG AATTGGTAAT AAACA 25

(2) INFORMATION FOR SEQ ID NO:42

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

AACACTACG 9

(2) INFORMATION FOR SEQ ID NO:43

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

GTGCTACTAG TTACTGTGTT 20

(2) INFORMATION FOR SEQ ID NO:44

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CACGTGGCGA GACCCTGGTA TGGGT 25

(2) INFORMATION FOR SEQ ID NO:45

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

TGCGACTATA TAATCCTAGG CGTGC 25

(2) INFORMATION FOR SEQ ID NO:46

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

TCTACAACTT TAACCTGTTG TGTTG 25

(2) INFORMATION FOR SEQ ID NO:47

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

AGTGGGAGTG GTTACAAAAG CAGGG 25

(2) INFORMATION FOR SEQ ID NO:48

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CAGGATTATC ATATGTAATA TTTGA 25

(2) INFORMATION FOR SEQ ID NO:49

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49

TTATCCACAT CTATACCTTC ATATG 25

(2) INFORMATION FOR SEQ ID NO:50

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50

ATCATTACTA GAAAAATATA ATGTA 25

(2) INFORMATION FOR SEQ ID NO:51

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51

GATCTGGAGC TATATTAATA CTATT 25

(2) INFORMATION FOR SEQ ID NO:52

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52

AAAGCAACTA TATCCAAAAA GTCAG 25

(2) INFORMATION FOR SEQ ID NO:53

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53

CCTAGAGGTT AATGCTGGCC TATGT 25

(2) INFORMATION FOR SEQ ID NO:54

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54

TTCTACTGTA CCTAATGCCA GTACG 25

(2) INFORMATION FOR SEQ ID NO:55

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55

CGTAGTGTTT GTTTATTACC AA 22

CLAIMS

Having thus described the invention, what it is desired to claim and thereby protect by Letters Patent is:

1. A process for detecting a two-stranded nucleic acid target, which process comprises the steps of

(1) separating the strands of the target sufficiently to allow them each to hybridize to a nucleic acid probe of complementary nucleotide sequence;

(2) co-incubating the sufficiently separated strands of the target with a nucleic acid probe population that comprises molecules complementary in nucleotide sequence to one target strand and molecules complementary in nucleotide sequence to the other target strand; and

(3) detecting the nucleic acid probe molecules that are hybridized to target molecules;

such that step (2) is performed under conditions that allow each strand of the target to form a hybrid with a nucleic acid probe molecule complementary in nucleotide sequence to that strand;

such that, as to the nucleotide sequence of each probe molecule, there is a totally complementary sequence in the target;

such that each probe molecule is partially complementary in nucleotide sequence to at least one other probe molecule;

such that no two probe molecules are completely complementary in nucleotide sequence to each other;

such that, where a portion of one probe molecule is complementary in nucleotide sequence to another probe molecule, that portion has a length which is too short to allow it to hybridize to the other probe molecule under the conditions of step (2).

2. A process of Claim 1 wherein the probe molecules have nucleotide sequences such that, if one strand of the target is saturated with probe molecules, then there will be no unhybridized target strand sequences forming gaps between the probe molecules.

3. A process of Claim 1 wherein the two target strands are located in a biological entity and wherein said biological entity is a cell or a virus.

4. A process of Claim 3 wherein the biological entity is a cell.

5. A process of Claim 4 wherein the cell is a prokaryotic cell.

6. A process of Claim 5 wherein the prokaryotic cell is a human cell.

7. A process of Claim 4 wherein the cell is a eukaryotic cell.

8. A process of Claim 4 wherein the cell is a plant cell.

9. A process of Claim 5 wherein the biological entity is a virus.

10. The process of Claim 3 wherein the biological entity is suspended in solution and not immobilized on a solid support.

11. The process of Claim 3 wherein the biological entity is immobilized on a solid support.

12. The process of Claim 3 wherein the biological entity is part of a tissue section or histologic section taken from a multicellular organism.

13. The process of Claim 1 wherein each of the probe molecules comprises a detectable label.

14. The process of Claim 13 wherein the detectable label is a fluorescent dye molecule.

15. The process of Claim 1 where the two target strands are purified nucleic acids.

16. The process of Claim 15 where the two target strands have been extracted from a virus, cell or multi-cellular organism.

17. The process of Claim 15 where the two target strands are immobilized on a solid support during Step (2) of the process.

18. The process of Claim 1 where the target strands are DNA.

19. The process of Claim 1 where the target strands are RNA.

20. A process of Claim 1 wherein each probe molecule is complementary in nucleotide sequence to a sequence, present in at least one other probe molecule, not less than about 12 nucleotides but not more than about 100 nucleotides in length.

21. A process of Claim 20 wherein each probe molecule is complementary to a sequence, present in at least one other probe molecule, not less than about 12 nucleotides but not more than about 20 nucleotides in length.

22. A process of Claim 21 wherein each probe molecule that is complementary to a sequence, present in at least one other probe molecule, is about 12 nucleotides in length.

23. A process of Claim 1 wherein the length of each probe is between about 15 nucleotides and 100 nucleotides.

24. A process of Claim 1 wherein the length of each probe is between about 15 nucleotides and 40 nucleotides.

25. A process of Claim 1 wherein the length of each probe is about 25 nucleotides.

26. A process of Claim 1 wherein the two-stranded target has a first target strand and a second target strand and wherein the probe molecules that are complementary in nucleotide sequence to the first target strand have a detectable label with a structure different from the detectable label on the probe molecules that are complementary in nucleotide sequence to the second target strand.

27. A process of Claim 26 wherein the detectable label on the probe molecules that are complementary in nucleotide sequence to the first target strand is a fluorescent dye and the detectable label on the probe molecules that are complementary in nucleotide sequence to the second strand is a fluorescent dye.

28. A process of Claim 26 wherein the two-stranded target is a DNA target and the wherein the probe molecules that are complementary in nucleotide sequence to the first target strand are also complementary in nucleotide sequence to cellular RNA molecules.

29. A process of Claim 28 wherein, for each of the two detectable labels that differ from each other, the amounts of label present in probe molecules hybridized to target molecules is determined and the amounts are used to determine the amounts of DNA that hybridized to the probes and the amounts of RNA that hybridized to the probes.

30. A process of Claim 1 where the target is viral DNA.

31. A process of Claim 1 where the target is viral RNA.

32. A nucleic acid probe population wherein

1) the length of each nucleic acid probe molecule is between about 15 nucleotides and about 100 nucleotides,

2) no probe molecule is totally complementary in nucleotide sequence to another probe molecule, and

3) each probe molecule is at least partially complementary in nucleotide sequence to at least one other probe molecule.

33. A kit for detecting a nucleic acid molecule in a biological entity, said kit comprising a probe population of Claim 32 and one more reagents for use in a solution for reacting said probe population with said biological entity so that a hybrid molecule can form between a molecule of the probe population and a nucleic acid molecule in the biological entity.

34. A kit of Claim 33 wherein the biological entity is a cell and the one or more reagents comprise a reagent selected from the group, a fixative and a chaotropic agent. (Preferred are the fixatives and chaotropic reagents identified in this application.)

Fig. 1

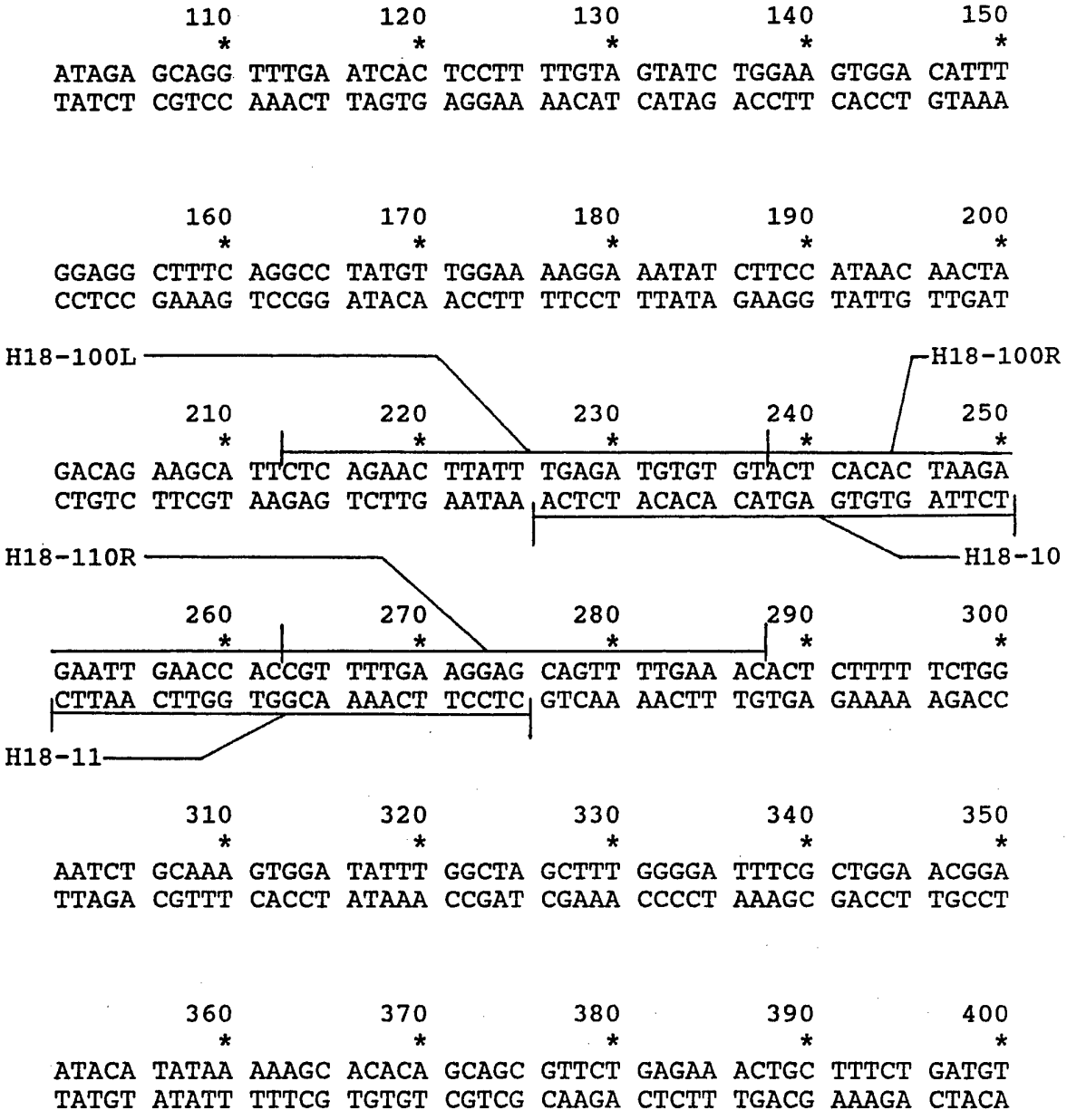
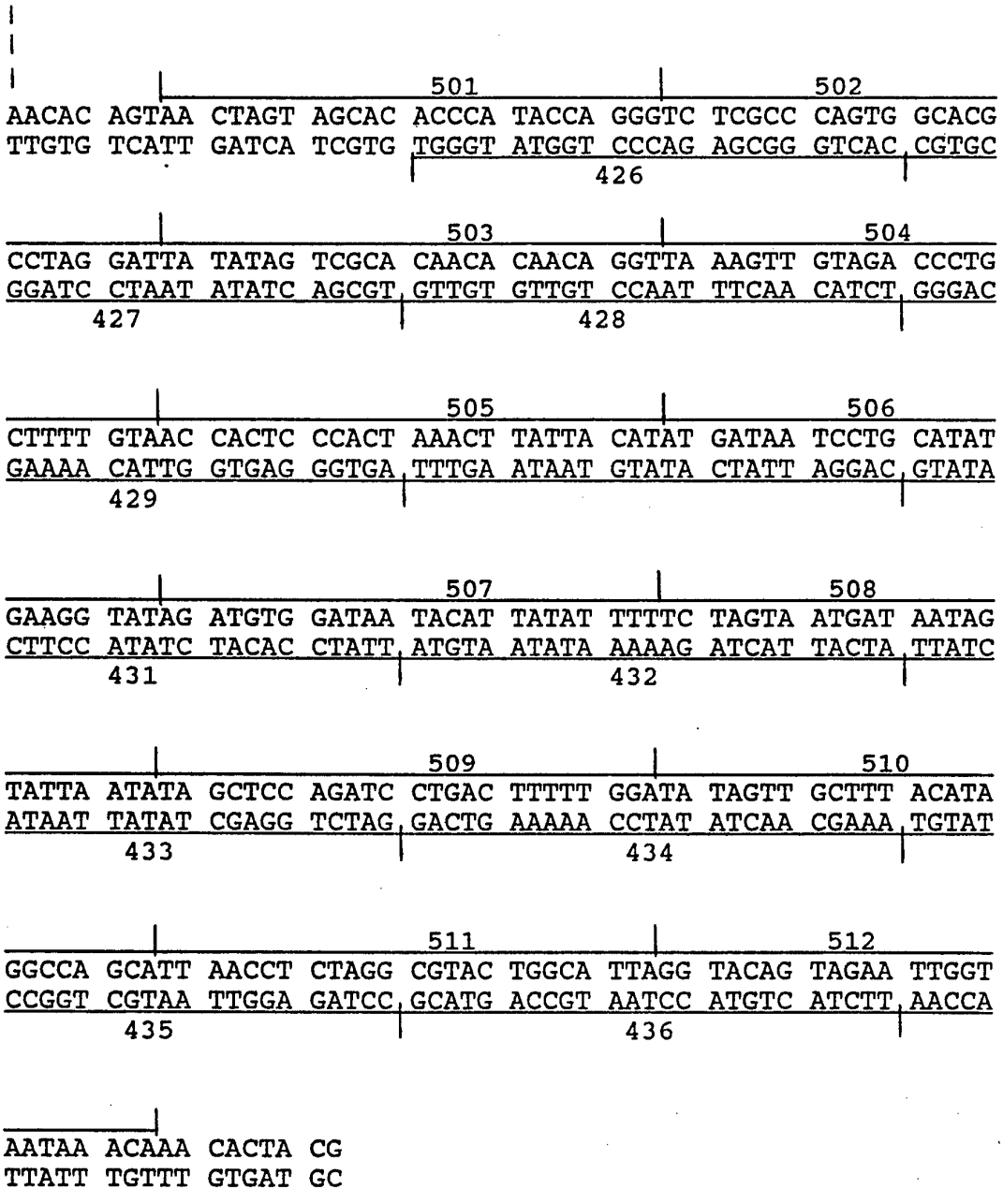

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GGAGG CTTTC AGGCC TATGT TGGAA AAGGA AATAT CTTCC ATAAC AACTA
CCTCC GAAAG TCCGG ATACA ACCTT TTCCT TTATA GAAGG TATTG TTGAT
H18-100L
H18-100R
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GACAG AAGCA TTCTC AGAAC TTATT TGAGA TGTGT GTACT CACAC TAAGA
CTGTC TTCGT AAGAG TCTTG AATAA ACTCT ACACA CATGA GTGTG ATTCT
H18-110R
H18-10
260 270 280 290 300
GAATT GAACC ACCGT TTTGA AGGAG CAGTT TTGAA ACACT CTTTT TCTGG
CTTAA CTTGG TGGCA AAACT TCCTC GTCAA AACTT TGTGA GAAAA AGACC
H18-11
310 320 330 340 350
AATCT GCAAA GTGGA TATTT GGCTA GCTTT GGGGA TTTCG CTGGA ACGGA
TTAGA CGTTT CACCT ATAAA CCGAT CGAAA CCCCT AAAGC GACCT TGCCT
360 370 380 390 400
ATACA TATAA AAAGC ACACA GCAGC GTTCT GAGAA ACTGC TTTCT GATGT
TATGT ATATT TTTCG TGTGT CGTCG CAAGA CTCTT TGACG AAAGA CTACA

Fig. 2


501
502
AACAC AGTAA CTAGT AGCAC ACCCA TACCA GGGTC TCGCC CAGTG GCACG
TTGTG TCATT GATCA TCGTG TGGGT ATGGT CCCAG AGCGG GTCAC CGTGC
426
503
504
CCTAG GATTA TATAG TCGCA CAACA CAACA GGTTA AAGTT GTAGA CCCTG
GGATC CTAAT ATATC AGCGT GTTGT GTTGT CCAAT TTCAA CATCT GGGAC
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428
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506
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GAAAA CATTG GTGAG GGTGA TTTGA ATAAT GTATA CTATT AGGAC GTATA
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508
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CTTCC ATATC TACAC CTATT ATGTA ATATA AAAAG ATCAT TACTA TTATC
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433
434
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512
GGCCA GCATT AACCT CTAGG CGTAC TGGCA TTAGG TACAG TAGAA TTGGT
CCGGT CGTAA TTGGA GATCC GCATG ACCGT AATCC ATGTC ATCTT AACCA
435
436
AATAA ACAAA CACTA CG
TTATT TGTTT GTGAT GC

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/06715

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :435/5, 6; 536/24.31, 24.32
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 91.2; 536/24.31, 24.32; 935/77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, MEDLINE, BIOSIS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A, 4,925,785 (WANG ET AL.) 15 MAY 1990, see entire document.	1-34
A	US,A, 4,868,105 (URDEA ET AL.) 19 SEPTEMBER 1989, see entire document.	1-34
A	ANALYTICAL BIOCHEMISTRY, Volume 138, issued 1984, J. Meinkoth et al., "Hybridization of Nucleic Acids Immobilized on Solid Supports", pages 267-284, see entire document.	1-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"E" earlier document published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 OCTOBER 1993	26 OCT 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PAUL B. TRAN, PH.D.
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A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

C12Q 1/68, 1/70; C07H 21/04