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(54) Title: cDNA-PHOTOFLUOR PROBE AND METHODS OF MAKING, ASSAYING AND USING SAME

(57) Abstract

Complementary DNA ("cDNA") probes that hybridize to specific DNA targets or consensus sequences, specifically a cDNA probe having attached thereto at least one fluorescent molecule (called a "photofluor" or "fluorophore") which fluoresces at a detectable wavelength (for example over autofluorescence) when suitably excited and assayed, and further to methods of making cDNA-photofluor probes, assay methods of determining positive binding between a cDNA-photofluor probe and its target consensus sequence, and methods of using cDNA-photofluor probes.

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cDNA-PHOTOFLUOR PROBE AND METHODS OF MAKING, ASSAYING AND USING SAME

The present invention relates to complementary DNA ("cDNA") probes that hybridize to specific DNA targets or consensus sequences, specifically to a cDNA probe having attached thereto at least one fluorescent molecule (called a "photofluor" or "fluorophore") which fluoresces at a detectable wavelength (for example beyond autofluorescence) when suitably excited and assayed, and further to methods of making cDNA-photofluor probes, assay methods of determining positive binding between a cDNA-photofluor probe and its target consensus sequence, and methods of using cDNA-photofluor probes.

BACKGROUND OF THE INVENTION

cDNA probes have been used to create restriction maps for determining the presence and location of specific DNA sequences. In particular, a given cloned gene (also called a restriction fragment) has been used as a probe to search out other DNA segments of identical or nearly identical sequences. Known methods of screening involve production of replicas or plaques of bacterial colonies on nitrocellulose filters, followed by treatment with sodium hydroxide (NaOH) to lyse the bacteria and denature the DNA which remains attached to the filter. The filter is then exposed to the specific radioactively labeled cDNA probes, and hybridization occurs. The hybridized sequences are then detected by their radioactivity.

Another screening technique involves Southern blotting (named after molecular biologist E.M. Southern) which commences with the cleavage of a DNA population with one or more restriction enzymes into restriction fragments which are then separated by agarose gel electrophoresis according to their size, and transferred to nitrocellulose sheets by use of a flow buffer so that an exact replica of

the DNA fragments that were present in the gel are present on the nitrocellulose filter. Radioactively-labeled cDNA probes having a specific sequence corresponding with targets on the fragments are then applied and hybridization occurs. Subsequent autoradiography of the filter results in a specific pattern of bands that correspond with the discrete restriction fragments that are complementary to the DNA probe.

Still further known uses of cDNA probes involve cloning portions of the population of mRNA molecules within specialized cells to isolate, e.g., the gene segments that code for exons away from their noncoding, intron components.

Such cDNA probes, and indeed DNA probes in general have heretofore been labeled with a radioisotope, using a number of known techniques. In particular, a given synthetic DNA probe, generally 20-50 bases (or "mer") in length are tagged with a ^{32}P label through use of the enzyme T4 polynucleotide kinase which transfers the label from ATP to a 5'-OH group on the DNA or RNA molecule (which can be an oligonucleotide, restriction fragment or mRNA, or any species having the 5'-OH group). DNA restriction fragments and RNA which have a 5'-phosphate terminus require dephosphorylation with a phosphatase enzyme before such labeling is performed. Another suitable method for radioisotope labeling on the 3'-end uses the enzyme terminal deoxynucleotidyl transferase.

Likewise there are a number of known methods for creating the sequence of a particular cDNA probe through DNA to RNA to cDNA synthesis (followed by denaturing), or through use of the so-called gene machines. In particular, the techniques invented by Caruthers and Beaucage, disclosed in U.S. Patent Nos. 4,415,732 and 4,458,066, incorporated herein by reference, are employed with the substitution of a pre-radioisotopically labeled nucleotide for the

manufacture of cDNA probes of over 100 mer. Of course, avoidance of problems involving stearic hindrance and conformational changes are considered when polynucleotides of such length are constructed. However, though cDNA-radioisotope labeled probes have been used and constructed, there are a plethora of intrinsic and unavoidable drawbacks caused by the incorporation of radioisotopes. Drawbacks generally inherent in radioisotope usage include high costs, potential health hazards both in preparation and in utilization, and difficulties in disposal of waste material. These drawbacks are aggravated when radioactive labeling is employed with complementary DNA ("cDNA"). Where the labeled probe is seeking a single, specific complement, as in the case of CDNA seeking a specific consensus or target sequence, and the sequence is present on an intra-nuclear DNA segment, the radioisotope tracing techniques are difficult to perform, often imprecise and fraught with potentially dangerous health hazards. In situations where the cell is left intact (i.e., not first lysed and the DNA cleaved with restriction enzymes), such difficulties arise as a consequence of one or a few single, hybridized DNA portions circulating in a large slurry of nuclear material.

One solution to this problem of detection employs the pre-placement of a large radioisotope "load" on the cDNA probe to make the autoradiographic spot of the single resultant hybrid appear larger. However, such a large load results in even greater emission of radiation, exacerbating the health situation, may prove fatal to the cell or act as an impediment to the cellular activity, thereby preventing the continued study of the cell activities, e.g., protein synthesis through DNA-RNA pathways. Alternatively, this large load may require that the host metabolism tolerate the presence of the loaded probes that are not bound, i.e. hybridized, to the target sequence.

Fluorescent dyes have been used in biology for many years as stains and labels, followed by the recent introduction of confined, attached fluorescent tags (called "photofluors") having a single or multiplicity of fluorescent molecules attached to cellular molecules (or macromolecule). Also, a number of very sensitive analytical procedures in chemistry and biology use reagents tagged with fluorescent dye molecules and detect and trace these by various spectrofluorometric or microspectrophotometric procedures.

It is thus an object of the present invention to provide a new cDNA probe that can function like the cDNA-radioisotope labeled probes (e.g., for restriction mapping, Southern blotting, sequencing, cellular isolation and the like) and is detectable, but does not possess the drawbacks associated with the use of radioisotope labelling and can be used to study the cellular events in question more precisely.

It is a further object of the present invention to provide a cDNA probe that can be used for intracellular detection of a target sequence without interfering with cellular activity.

It is a still further object of the present invention to provide a method of making a new cDNA probe that is assayable but does not have a radioisotopic label.

It is another object of the present invention to provide a method of incorporating a new cDNA probe lacking a radioisotopic label into a cell.

It is yet another object of the present invention to provide a method of assaying a cDNA probe and its attachment to a target sequence without the use of autoradiography.

SUMMARY OF THE INVENTION

The foregoing objects, and other objects of the instant invention are achieved by the provision of a cDNA probe labeled with

at least one fluorescent molecule selected from the group consisting of 5- (and 6-) carboxyfluorescein-N-hyroxysuccinimide, 7-hydroxycoumarin-4acetic acid, acridone-10-acetic acid, tetramethylrhodamine-5- (and 6-), isothiocyanate (TRITC), and 5- (and 6-) carboxymethylrhodamine-N-hydrosuccinimide ester, and preferably tetramethethylrhodamine (herein referred to as "rhodamine".) The objects are further achieved by the provision of methods of making such a probe by attaching the fluorescent molecule(s) to a pre-engineered cDNA fragment, to methods of incorporating the cDNA-photofluor probe into a target cell or into the nucleus to address its DNA target, and to post-hybridization assay methods.

Accordingly, it is a feature of the instant invention that cDNA-photofluor probes overcomes many of the disadvantages present in radioactive probes, while resulting in many advantages, including ease of detection, lack of risks in manufacture, ability to incorporate into cellular activity without interference, and safety.

It is another feature of the instant invention that one or more photofluors are attached to a cDNA probe, which fluoresce at a detectable level of emission intensity.

It is a further feature of the instant invention that a photofluor-cDNA probe can be readily made.

It is still a further feature of the present invention probe that simple assay techniques can be employed to determine positive hybridization.

DETAILED DESCRIPTION OF THE INVENTION

Complete comprehension of the subject invention is achieved by reference to the various preferred embodiments set forth under the particular subheadings below.

cDNA-photofluor Probes and Methods of Making Them

In accordance with the invention, cDNA of between about 10 and 100 mer are attached to a photofluor molecule or group of molecules. Virtually any fluorescent molecule may be used that fluoresces above autofluorescence (the natural fluorescence of the target or system) and can be attached to the 3' or 5' end of the probe. Acceptable dyes include 5- (and 6-) carboxyfluorescein-N-hyroxysuccinimide, 7-hydroxycoumarin-4-acetic acid, acridone-10-acetic acid, tetramethylrhodamine-5- (and 6-), isothiocyanate (TRITC), and 5- (and 6-) carboxymethylrhodamine-N-hydrosuccinimide ester. The preferred photofluor is rhodamine since it is excited at 570nm and read at 610nm, and thus can be excited by an LED, while observed well above autofluorescence (UV wavelengths) in the appropriate spectrum (e.g., visible).

In accordance with the invention, a photofluor molecule or group of molecules is first attached to a single base, and the base then used to form a sequence of DNA. Alternatively, a strand of DNA can first be formed and the photofluor thereafter added.

A particular sequence of DNA is synthesized by way of a number of methods. For example, long strings of DNA are synthesized using the automated gene machines created by Caruthers and Beaucage (and commercially available through Applied Biosystems, Inc.).

Alternatively, double stranded DNA is first synthesized and then denatured into a single stranded cDNA probe. For example, using the enzyme reverse transcriptase, sequences in mRNA are copied into cDNA replicas. Reverse transcriptase requires a primer (onto which subsequent nucleotides are added) which is added via a number of techniques known by those skilled in the art. For example,

cDNA is primed by utilizing a so-called "poly A tail" (i.e., multiple adenine bases) attached to the 3' end of an mRNA molecule having the matching sequence for transcription to the cDNA probe. Oligo dT primer is added thereby making a complementary thymine tail, which acts as the primer for subsequent transcription using reverse transcriptase. A single stranded cDNA molecule is thereby created, running to the 5' end of its RNA template. At the 5' end, the DNA chain will likely make a hairpin loop turn, which can serve as a primer for synthesis of the cDNA (secondary) complement to the cDNA (primary) formed from the RNA template, thereby making a double-stranded DNA molecule. The mRNA is degraded using NaOH. The cDNA secondary is formed using DNA polymerase I. Lastly, a specific nuclease (specific to sever the hairpin of the single stranded DNA) is used to eliminate the hairpin whether single or double stranded cDNA is required. Alternatively, the hairpin is used as a location for attachment of the photofluor molecule. A similar procedure is disclosed in McCabe, et al. "Characterization of Benzodiazepine Receptors with Fluorescent Ligands", The FASEB Journal, Vol. 4, Aug. 1990, pp. 2934-2940, and incorporated herein by reference.

The cDNA can be purified using known techniques, including electrophoresis and column chromatography. However, because of the sensitivity of many photofluors to light, fluorescent labeling is generally performed under reduced light. Such techniques, as well as methods for equipping the laboratory for fluorescent analog cytochemistry, are set forth in Wang, Yu-Li, Methods in Cell Biology, Vol. 29, Chptr. 1, pp. 1-12 (1989).

A photofluor molecule, or group of photofluor molecules linked together, are then coupled to the cDNA probe through known coupling pathways at either the 3' or 5' end. For example, the

carboxyl group can be bonded to an NH_2 terminal group on the rhodamine molecule by extraction of a water molecule. In this manner, a cDNA-photofluor probe is synthesized.

Methods of Incorporating cDNA-photofluor Probes

Though cDNA-photofluor probes can be used in much the same ways as probes heretofore labeled with a radioisotope, i.e., for restriction mapping, Southern blotting, and the like, they are also suitable for loading into intact cells where they can migrate towards intranuclear DNA and their respective consensus sequences.

Loading the targeted cells involves four strategic mechanisms for breaching the cell plasma membrane: chemical, vehicular, mechanical and electrical. Of course, overlaps between these strategies will be known by those skilled in the art since, for example, there is typically a mechanical element in every successful strategy, owing to the forcing apart of membrane components for entry of macromolecules in general and the cDNA-photofluor probes in particular. For example, short wavelength and small pulse duration eximer lasers can be used to temporarily and reversibly perforate the membrane. Various forms of entry techniques are taught by McNeil, Paul J. Incorporation of Macromolecules, Chapter 10, p. 155, Table I, which is incorporated herein by reference.

In the chemical mechanism, the particular targeted cells are rendered permeable by adding chemical agents like ATP or EGTA to the medium. Diffusion (passive, by gradient) across the cell membrane follows by the placement of a volume of probe in the medium. Under an alternative embodiment, $CaPO_4$ and DEAE-dextran treatment are used to achieve transfection with the cDNA-probe. Mechanisms for transport responsible for this treatment may involve forming a complex of the DNA probe with $CaPO_4$ or

DEAE, which enters the cytoplasm either directly, or first by pinocytosis later being released into the cytoplasm.

In the vehicular mechanism, cells are loaded by causing them to fuse with a suitable membrane-delimited vehicle (typically a red blood cell ghost or liposome) which has been previously loaded with the probe. Efficiency of delivery by red blood cell ghosts is enhanced by the placement of fusogenic viral hemagglutin on the target cell. The viral hemagglutin improves fusion, and therefore loading of adherent cells. This action is triggered by reducing medium pH.

The electrical mechanism (called "electroporation") is the preferred method of loading. Under this method, cells are permeabilized, often reversibly, to the exogenous cDNA-photofluor probes by high voltage electric discharge. In this method, the targeted cells and linearized cDNA-photofluor probes are placed in a foil-lined plastic cuvette properly configured for electroporation.

Aluminum foil electrodes are prepared having a thickness of 1 mil, and are inserted in the opposite ends of the cuvette. A discharge switch that is suitable for high voltage and possesses the necessary shielding is placed in the circuit. Before insertion of the cells and probe, the chamber of the cuvette is sterilized with alcohol. Devices of this type can be obtained from Bio-Rad and Prototype Design Services (Madison, WI).

The cells are prepared for insertion into the electroporation cuvette, and electroporation is performed in ways known to those skilled in the art. In particular, the cells are harvested, then concentrated by centrifugation, the medium is removed and the pelleted cells are resuspended in phosphate-buffered saline (PBS) (e.g., 5 ml. at 4°C), chilled, and transferred to the cuvette on ice. The cDNA probe, which is linearized by, e.g., RE digestion, is then

added. A shock is applied of 2,000 V, 0.9mA, at 5 %. After sitting for 10 min. on ice, the cells are transferred under sterile conditions to a cell culture plate or flask, and growth medium (10 ml at 4°C) is added.

After a period of time elapses from presentation of cDNA-photofluor probes in excess quantity to enable gradient-based diffusion, the extracellular medium is washed, such that non-intracellular probes are removed. Now, the gradient direction switches and by passive diffusion, unbound, intracellular cDNA-photofluor conjugates flow outwardly through the cell membrane. After a certain predetermined time passes, the extracellular fluid is again washed. Likewise, unbound, intranuclear cDNA-photofluor will also leave the cell through normal diffusion and be washed away.

An alternative method for removing unconjugated fluorophores involves chemical removal through, e.g., the Bio-Bead SM2 (Bio-Rad Laboratories, Richmond, California). These beads remove free fluorophores based on hydrophobic interactions and are suitable for use with fluorescein or tetramethylrhodamine, and are simply added to the extracellular medium. As the gradient changes (free conjugates become adsorbed into the bead), intracellular conjugates are released. Likewise, ion-exchange chromatography may be used to separate adsorbed probes, since, in some cases, it has the advantage of fractionating conjugates according to the degree of labeling.

Yet an alterative method for extraction of unbound cDNA-photofluor probes from bound conjugates involves lysing the cells by, e.g., introducing a hypotonic environment (like water). Thereafter, unbound and nonspecifically bound probes can be washed away using a physiologic medium. Alternatively, electrophoretic

techniques may be employed, owing to the differences in weight/charge distribution accompanying the binding of captured DNA to the cDNA-photofluor probe. These techniques are followed by microscopic inspection, and spectrophotometry and/or spectrofluorometry for excitation and emission spectra.

Assay Techniques for cDNA-photofluor Probes

Assay techniques will provide single photofluor reading. Elimination of the false positive situation wherein a cDNA-photofluor is bound to an intracellular non-DNA component, is achieved through, e.g., dual fluorescent microscopy. Thymidine autofluoresces at a wavelength in the ultraviolet region, and thus can be separated from the fluorescence of rhodamine, which is at a significantly higher wavelength. Since DNA is approximately one fourth thymidine, spectrophotometric and/or spectrofluorometric analysis will entail recognizing a close proximity between the probe and large thymidine-containing DNA. Alternatively, dual dyes may be used to achieve the same result. One skilled in the art will readily recognize these techniques, in light of the teachings herein, and be capable of proper application to the particular situation.

There are a number of methods contemplated and incorporated herein for increasing the fluorescent signal emanated from the tagged DNA. For example, enhancement of the signal to nose ratio, and thereby improving fluorescent detection of the hybridized cDNA-photofluor probe, may be achieved. In particular, and as a practical matter, it is generally desirable to link a number of fluorescent molecules to the probe (thereby attaching to the target), to increase the fluorescent molecular population and the light intensity (when excited), thereby simplifying detection and enabling real-time detection. Care must be taken to avoid concentration quenching when a polyethyleneamine oligomer (having, e.g., a MW of 20000) or other

binding backbone is used (a "kite tail") and bound with, e.g., a bridge to the probe.

Ideally, the fluorescence should be so intense that real-time detection is possible. This can be accomplished by illuminating the target (solution, surface if on a filter, etc.) with a special (narrow band) light source and viewing the fluorescence through a narrow band filter which effectively blocks the illuminator. This, the simplest technique for enhancing the fluorescence, is frequency separation. For example, the preferred photofluor rhodamine is excited at 570nm and read at 610 nm. Again, the illumination source is kept well below the brightness level that leads to photochemical destruction or bleaching of the rhodamine molecules. The formulas and other information necessary to determine the level at which bleaching occurs are contained in Hirschfeld, T., "Quantum Efficiency Independence of the Time Integrated Emission from a Fluorescent Molecule," Applied Optics, Vol. 15, No. 12 (December 1976), at pp. 3135-3139, the text of which is incorporated herein by reference.

However, where the cDNA-photofluor probe has but a single fluorescent molecule (as in an instance where multiple rhodamine molecules render the probe too large), detection is still possible using signal averaging. In this case, the target is strobe illuminated, the fluorescence is imaged, the bins, i.e., the intensity of each pixel in the image, are counted, and the result is stored. The process is repeated many times, with the digital cumulative sum (per pixel) divided by the number of cycles the process has been repeated. Random noise increases in this "normalized" environment as the square root of the number of trials, while a real signal will increase

almost in direct proportion to the number of trials. Therefore, the signal to noise ratio will improve as nearly as:

$$\frac{\sqrt{N}}{N}$$

This is also referred to as a random walk (or drunkard's walk) signal enhancement technique. Of course, care must be taken not to repeatedly photoexcite to the point of bleaching the rhodamine molecule.

Using a machine almost as simple as a standard home video camera having, e.g., a one millisecond "shutter" time, i.e. the charge coupled device ("CCD") image is clocked for 1 msec., and assuming a normal frame rate of 30 frames/second ("fps"), it takes just over five and one-half minutes to accumulate 10,000 samples (which can be digitized, added, stored, and "normalized" by dividing each store total by the frame number). This gives a signal to noise ration enhancement of almost 100 to 1, which is very nearly the equivalent to increasing the fluorescent molecular population by 100, and is preferred. By way of example, in nine and one quarter hours the improvement approaches 1000 to one.

If the target is a transcriptively active site on a gene, then the cDNA-photofluor probe will, within a certain period of time, bind with its target. Bound cDNA, as a consequence of its affinity, will move less randomly over time than unbound cDNA. Consequently, if timedelay averaging is used, through, e.g., photomicrography or any other mechanism that gives quantities and positioning, the more random, unbound cDNA will move with greater degrees of freedom, and therefore will give repeating sequences from one photomicrograph to the next. Accordingly, by regional summing, confirmation of hits (v. non-binding) is achieved.

The following examples further illustrate the invention.

EXAMPLE I

The Borna virus has been associated with an infectious disease of horses and sheep in central Europe. A recently developed cDNA probe specific to the integrated DNA sequence of the virus has shown its presence, by radioisotope tracing, in a significant number of institutionalized patients with behavioral disorders ranging from aggression to apathy to "schizoid" manifestations.

In this example, the virus may be presented in sufficient concentration in easily accessible cells, such as macrophages, for in vitro testing. A suitable cDNA-rhodamine probe may be synthesized using the techniques set forth above. This probe may be contained in a kit form for use in a clinical setting, including a simple detection mechanism using an LED and reader. This special light source presents a resonance line ideal for examination. Fluorescent examination for cDNA hybridization may be performed using the techniques set forth above. Except for obtaining a blood (or appropriate tissue) sample, this can be a non-invasive, safe, convenient and definitive test, external to the patient and independent of special site licenses and facilities.

EXAMPLE II

There is a relatively stable consensus sequence in the H.I.V. viral coat protein, g.p. 120, which is coded for by an integrated DNA sequence. A cDNA-rhodamine probe for this or other H.I.V. sequences would provide direct proof of infection much earlier and more reliably than the currently used antibody assay which misses the silent (i.e., post-infection but pre-antibody) period, and still permits significant contamination of the blood supply, and makes early detection difficult, due to the cost and complexity of current antigen test methods.

EXAMPLE III

Many genetic defects now have known DNA sequences or DNA defects. Hybridizing to a known defect sequence or, perhaps more importantly, poor hybridization to a sequence subject to mutational or inherited defects directly identifies many genetic abnormalities, either early in life, before the defect was manifest (Huntington's chorea, for example), or even during fetal development (by amniocentesis or puncture biopsy), giving early warning or providing early reassurance.

EXAMPLE IV

Using a cDNA-photofluor probe with a molecule having a distinctive magnetic resonance signature, hybridization may be allowed to occur in search of metastatic colonies. The cDNA sequence will be selected to hybridize with a consensus sequence unique to a malignant cell population contained within the cell culture. Unbound cDNA probes are removed by reverse diffusion washing (i.e., after a period of time sufficient for gradient diffusion into the cells and hybridization, the cells are washed, and unbound cDNA probes diffuse back into the medium, followed by an additional washing). Through MRI scanning of the culture thereafter, conformation of presence or absence of the metastatic colonies can be achieved.

EXAMPLE V

This example involves the use of a chemical mechanism for uptake of a cDNA-rhodamine probe, using serum-free Dulbecco-Vogt modified Eagle's medium (SFM). In this case, targeted cells will be rinsed gently twice with SFM at 37°C. Where concentration dependence studies are sought, various concentrations of cDNA-rhodamine probes can be added to the cells in 1 ml. of SFM, and the cells incubated at 37°C for 30 min. At the end of incubation, the cells can be rinsed 4 times with warm SFM and once in

Dulbecco's phosphatebuffered saline (PBS). The cells can then be placed for 5 min. in 2% formaldehyde in PBS and rinsed in PBS. Mounting fluid (DIFCO) can then be put on the fixed cells and a no. 1 cover slip placed thereover. The cells can then be tested for fluorescence using the techniques set forth above.

Accordingly, while there have been shown, described and pointed out the fundamental novel features of the invention as applied to preferred embodiments thereof, it will be understood that various omissions, substitutions and changes in the form and details illustrated and in the operations and methods employed may be made by those skilled in the art without departing from the spirit of the invention. It is the intention, therefore, to be limited only as indicated by the scope of the claims appended hereto.

We Claim:

1. A cDNA probe having attached thereto at least one photofluor molecule selected from the group consisting of 5- (and 6-) carboxyfluorescein-N-hyroxysuccinimide, 7hydroxycoumarin-4-acetic acid, acridone-10-acetic acid, tetramethylrhodamine-5- (and 6-), isothiocyanate (TRITC), and 5- (and 6-) carboxymethylrhodamine-N-hydrosuccinimide ester.

2. The cDNA probe of claim 1 wherein said photofluor molecule is tetramethylrhodamine.

3. The cDNA probe of claim 1 having between 2 and 150 mer.

4. The cDNA probe of claim 1 wherein a plurality of photofluor molecules
are attached to a backbone which is in turn attached to the probe.

5. The cDNA probe of claim 1 wherein the photofluor molecule fluoresces at a wavelength sufficient to be detectable and distinguishable from autofluorescence.

6. A targeted cell having a hybridized DNA consensus sequence comprising at least one cDNA probe having attached thereto at least one fluorescent molecule within the cell.

7. A method of loading targeted cells with cDNA probes, comprising:

preparing cDNA probes having at least one fluorescent molecule attached per probe;

placing said targeted cells in a first medium;

placing said cDNA probes in said medium at a concentration sufficient to provide a diffusion gradient into the cells;

rendering a plurality of cell membranes of said targeted cells permeable to said cDNA probes; and

allowing a first period of time to pass sufficient to provide for diffusion of said cDNA probes through said cell membranes into the cytoplasm of said cells.

8. The method of claim 7 wherein said targeted cells each have a nucleus containing intranuclear DNA, and said first period of time for diffusion is sufficient to allow for:

diffusion of at least one of said cDNA probes across the nuclear membrane of at least one of said targeted cells; and

hybridization of said cDNA probe if a complementary target sequence portion of at least one fragment of intranuclear DNA is present.

9. The method of claim 8, further comprising the steps of:

transferring said cells to a second medium that contains no cDNA probes;

allowing a second period of time to pass sufficient to provide for diffusion of unhybridized intranuclear cDNA probes into said cytoplasm, and intracellular cDNA probes into said second medium;

transferring said targeted cells from said second medium to a third medium.

10. The method of claim 9, further comprising the steps of assaying said targeted cells transferred to said third medium to determine whether hybridization has occurred.

11. The method of claim 7, wherein said step of rendering said cells permeable is achieved by mechanical means.

12. The method of claim 11, wherein said mechanical means employs a short wavelength and small pulse duration eximer laser for perforation of said cell membranes.

13. The method of claim 7, wherein said step of rendering said cells permeable is achieved by chemical means.

14. The method of claim 7, wherein said step of rendering said cells permeable is achieved by vehicular means.

15. The method of claim 7, wherein said step of rendering said cells permeable is achieved by electrical means.

16. The method of claim 15, wherein said electrical means is an electroporation method.

17. The method of claim 16, wherein said electroporation method comprises the following steps:

placing said cells and probes in said first medium into a cuvette having electrodes at either end thereof; and

applying a power supply to said electrodes at a wattage and for a period of time sufficient to porate said cells without causing cellular destruction.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04234

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; CO7H 15/12
US CL :435/6; 536/27

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. : 435/6; 536/27

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)

APS and DIALOG files MEDLINE, Biotechnology Abstracts and World Patents. Keywords searched: (In situ hybridization and fluorescence) or FISH; TRITC or tetramethylrhodamine.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 4,855,225 (S. Fung et al) 08 August 1989, columns 7, 8.	1-4 5-17
Y	Cytometry, Volume 10, issued 1989, P. M. Nederlof et al., "Three-Color Fluorescence In Situ Hybridization for the Simultaneous Detection of Multiple Nucleic Acid Sequences", pages 20-27, especially pages 20 and 21.	6-10
Y	Experimental Cell Research, Volume 162, issued 1986, S. Kurata et al, "The Laser Method for Efficient Introduction of Foreign DNA into Cultured Cells", pages 372-378, especially page 375.	11,12
Y	Biochemical and Biophysical Research Communications, volume 147, No. 3, issued 30 September 1987, C. Wang et al, "Plasmid DNA Adsorbed to pH-sensitive Liposomes Efficiently Transforms the Target Cells", pages 980-985, entire document.	14
Y	Kreigler, "Gene Transfer and Expression: A Laboratory Manual", published 1990 by Stockton Press, (New York, N.Y.), pages 101-102, entire document.	15-17

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

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