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MICHAEL W DOBBINS
CLERK, U.S. DISTRICT COURT

IN THE UNITED STATES DISTRICT COURT
FOR THE NORTHERN DISTRICT OF ILLINOIS
EASTERN DIVISION

Ventana Medical Systems, Inc.,

Plaintiff

v.

Vysis, Inc. and
Abbott Laboratories,

Defendants.

ARCH Development Corporation,

Necessary Party

03C 4870

Case No.
JUDGE LINDBERG

MAGISTRATE SIDNEY I. SCHENKIER

Jury Trial Demanded

DOCKETED
JUL 1 6 2003

COMPLAINT

This is an action for patent infringement brought by Ventana Medical Systems, Inc. ("Ventana") against Vysis, Inc. ("Vysis") and its parent company, Abbott Laboratories ("Abbott"). ARCH Development Corporation ("ARCH") is joined as a necessary party pursuant to Rule 19(a) of the Federal Rules of Civil Procedure.

Parties

1. Plaintiff Ventana is a corporation organized under the laws of Delaware, with a principal place of business at 1910 Innovation Park Drive, Tucson, Arizona. Ventana is the exclusive licensee, with rights to enforce, United States Patent No. 6,025,126 (" '126 patent"), entitled "Methods and Compositions for the Detection of Chromosomal Aberrations," issued February 15, 2000. Ventana is the assignee of title to United States Patent No. 6,414,133 (" '133

patent"), entitled "Multiple Fusion Probes," issued July 2, 2002. True copies of the '126 and '133 patents are attached as Exhibits 1 and 2.

2. Defendant Vysis is a Delaware corporation with a principal place of business in Downers Grove, Illinois. Among other things, Vysis manufactures, offers to sell, and sells cellular genomic products for hematopoietic disorders. Vysis is a wholly-owned subsidiary of Abbott.

3. Defendant Abbott is a corporation organized under the laws of the state of Illinois, with a principal place of business in Abbott Park, Illinois. Abbott is a health care company in the business of manufacturing, offering to sell and selling, among other things, diagnostic tests, pharmaceuticals and medical products.

4. ARCH is an Illinois not-for-profit corporation with a principal place of business in Chicago, Illinois. ARCH is the assignee of title to the '126 patent. ARCH is subject to service of process, and is joined as a necessary party pursuant to Rule 19(a) of the Federal Rules of Civil Procedure as a prudent measure to ensure complete adjudication of rights relating to the patent.

Jurisdiction and Venue

5. This is a Complaint for patent infringement arising under 35 U.S.C. § 271. This Court has subject matter jurisdiction over this matter under 28 U.S.C. § 1331 and 28 U.S.C. § 1338(a).

6. Venue is proper in this judicial district under 28 U.S.C. §1391 (b) and (c) because both Vysis and Abbott reside in this judicial district and are subject to personal jurisdiction in this judicial district.

7. Venue is also proper in this judicial district under 28 U.S.C. § 1400(b) because both Vysis and Abbott reside in this judicial district and because Vysis and Abbott have committed acts of infringement and maintain established places of business in this judicial district.

8. Joinder of ARCH as a necessary party neither: (a) destroys jurisdiction of the subject matter of the action, nor (b) renders venue improper.

COUNT I

Infringement of the '126 Patent

9. Defendants have infringed and continue to infringe the '126 patent by making, using, selling and offering to sell certain products including, but not limited to, the "LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191032), "LSI BCR/ABL ES Dual Color Translocation Probe" (Vysis Catalog number 32-191022), and the "LSI BCR/ABL Dual Color, Single Fusion Translocation Probe" (Vysis Catalog number 32-190022).

10. Ventana makes, offers to sell, and sells a product called "BCR/ABL1 D-FISH probe" that competes with the Defendants' products listed in paragraph 9 such that each sale made by Defendants is a sale that rightfully should have been made by Ventana.

11. Defendants have continued their infringement despite having notice of the '126 patent. Defendants have committed, and are committing, willful patent infringement.

12. Defendants' infringement has caused monetary damage and irreparable injury to Plaintiff. Unless, and until, Defendants' infringement is enjoined by this Court, it will continue to cause monetary damage and irreparable injury to Plaintiff.

COUNT II

Infringement of the '133 Patent

13. Defendants have infringed and continue to infringe the '133 patent by making, using, selling and offering to sell certain diagnostic tests including, but not limited to, the "LSI AML1/ETO Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191006), "LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191032), "LSI IGH/MYC, CEP 8 Tri-Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191020), "LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191018), "LSI IGH/CCND1 Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191017), and, "LSI PML/RARA Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191013).

14. Ventana makes, offers to sell, and sells a product called "BCR/ABL1 D-FISH probe" that competes with the Defendants' "LSI BCR/ABL Dual Color, Dual Fusion Translocation Probe" (Vysis Catalog number 32-191032) product listed in paragraph 13 such that each sale made by Defendants is a sale that rightfully should have been made by Ventana.

15. Defendants have continued their infringement despite having notice of the '133 patent. Defendants have committed, and are committing, willful patent infringement.

16. Defendants' infringement has caused monetary damage and irreparable injury to Plaintiff Ventana. Unless, and until, Defendants' infringement is enjoined by this Court, it will continue to cause monetary damage and irreparable injury to Plaintiff.

Prayer For Relief

WHEREFORE, Plaintiff Ventana requests the following:

A. Award all damages adequate to fully compensate Ventana for the infringement that has occurred, including, but not limited to:

i. lost profits;

ii. reasonable royalty;

iii. prejudgment and post-judgment interest; and,

iv. any further damages permitted by 35 U.S.C. § 284.

B. Award enhanced damages, including treble damages, for willful infringement pursuant to 35 U.S.C. § 284.

C. Award attorneys' fees and other costs based on this being an exceptional case pursuant to 35 U.S.C. § 285.

D. Grant preliminary and permanent injunctive relief against Defendants', their customers, and all those acting in concert or participating with them from further acts of infringement of the `126 and `133 patents pursuant to 35 U.S.C. § 283.

E. Award such other and further relief as is just.

Jury Demand

Plaintiffs demand a jury trial on all issues so triable.

Dated: July 15, 2003

Respectfully submitted,

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Counsel for Ventana Medical Systems, Inc.

EXHIBIT 1

US006025126A

United States Patent [19]

Westbrook

[11] **Patent Number: 6,025,126**

[45] **Date of Patent: Feb. 15, 2000**

[54] **METHODS AND COMPOSITIONS FOR THE DETECTION OF CHROMOSOMAL ABERRATIONS**

[75] Inventor: **Carol A. Westbrook**, Chicago, Ill.

[73] Assignee: **Arch Development Corporation**, Chicago, Ill.

[21] Appl. No.: **07/784,222**

[22] Filed: **Oct. 28, 1991**

[51] **Int. Cl.**[7] **C12Q 1/68**; C12P 19/34; C07H 21/02; C07H 21/04

[52] **U.S. Cl.** **435/6**; 435/91.2; 536/23.1; 536/24.3; 536/24.32; 536/26.6

[56] **References Cited**

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Primary Examiner—Jeffrey Fredman
Attorney, Agent, or Firm—Arnold White & Durkee

[57] **ABSTRACT**

This invention relates generally to methods and compositions for direct detection of specific nucleic acid flanking sequences associated with structural chromosomal aberration breakpoints, by forming hybrids between the sequences and genetic probes, and detecting the probes. In particular aspects, the invention concerns detection of nucleic acid sequences in situ in chromosomes, and more specifically in cells, including interphase cells. Compositions of probes useful for detecting chromosomal translocations, in particular those associated with human leukemias, are also disclosed. An aspect of the invention is labelled probes that, when juxtaposed by formation of an aberration, are distinguishable and provide a pattern different from that of normal cells.

25 Claims, 5 Drawing Sheets

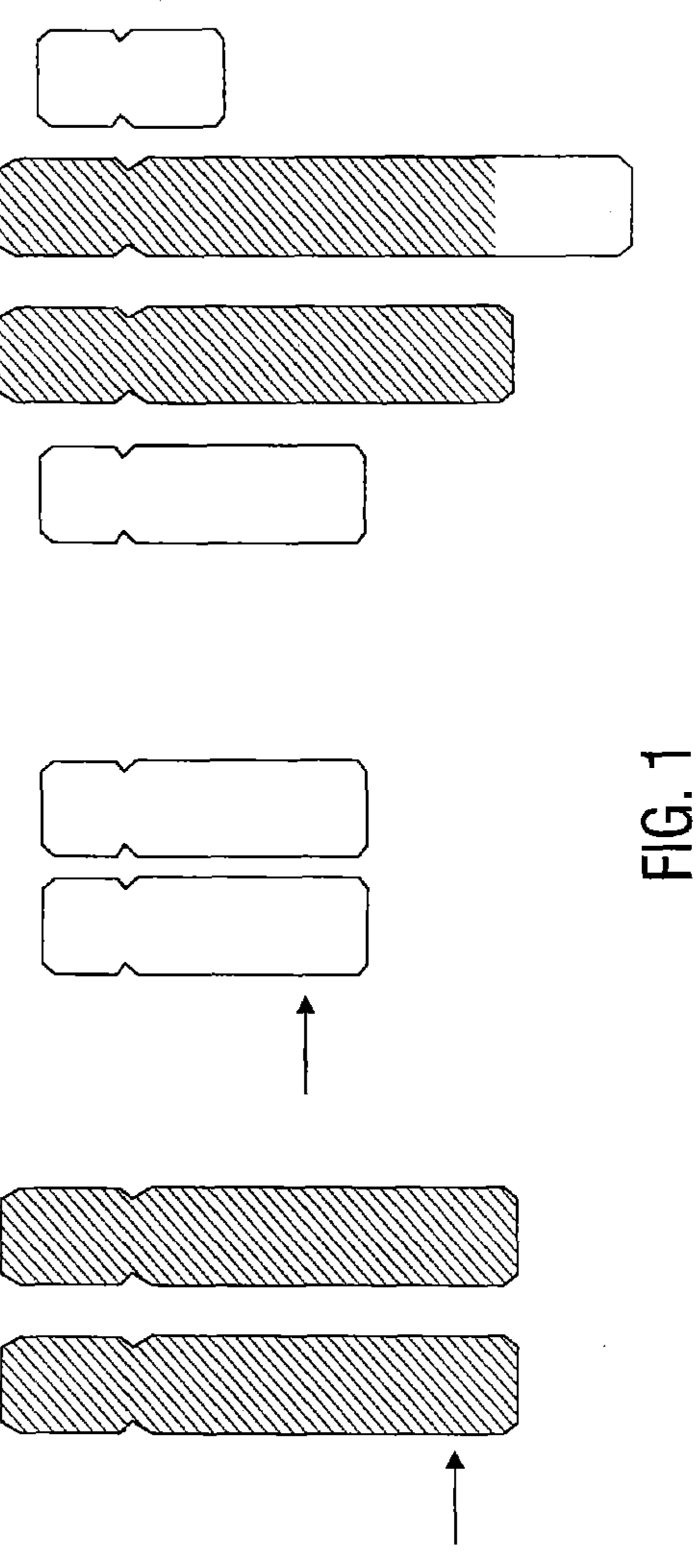

FIG. 1

Case: 1:03-cv-04870 Document #: 1 Filed: 07/15/03 Page 10 of 51 PageID #:10

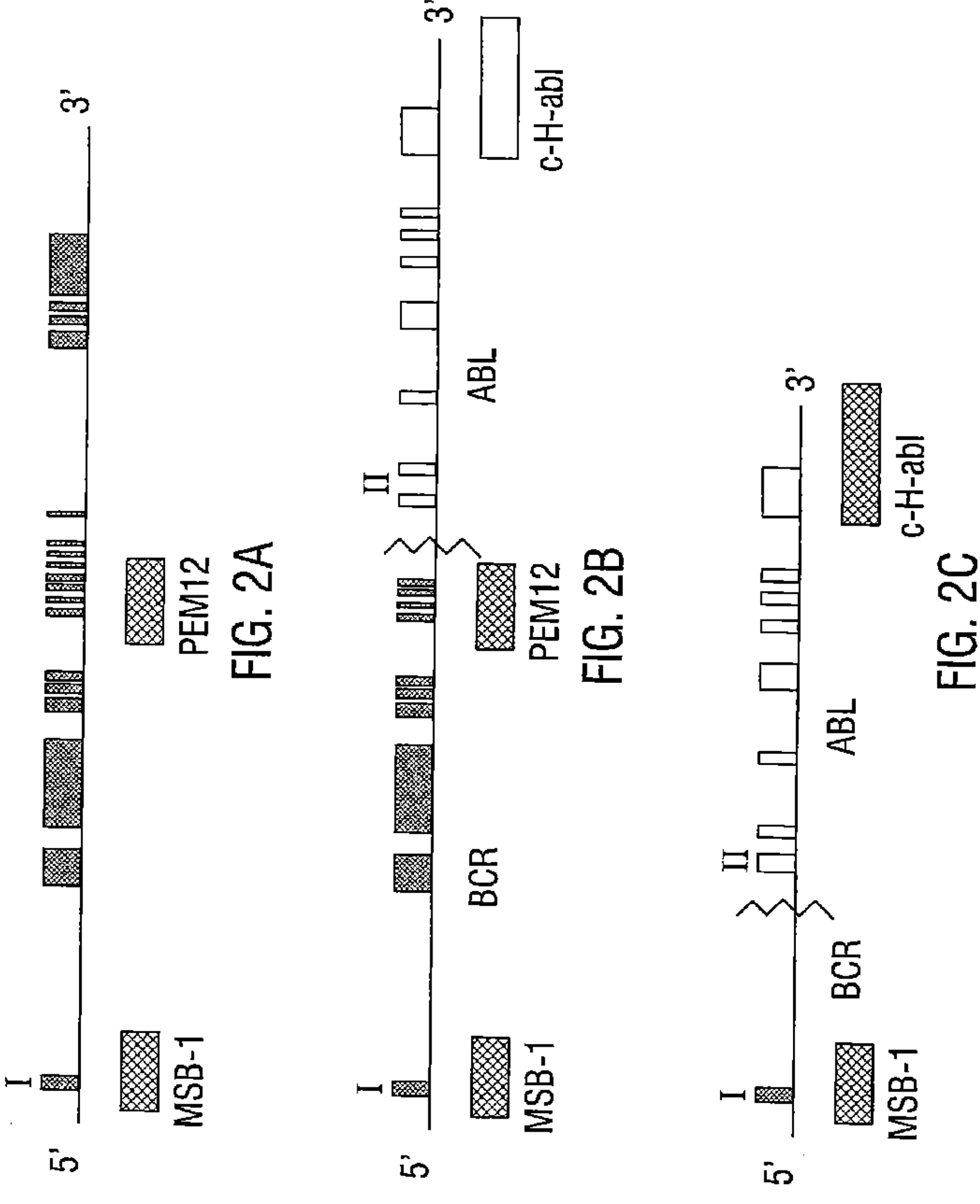

FIG. 2A

FIG. 2B

FIG. 2C

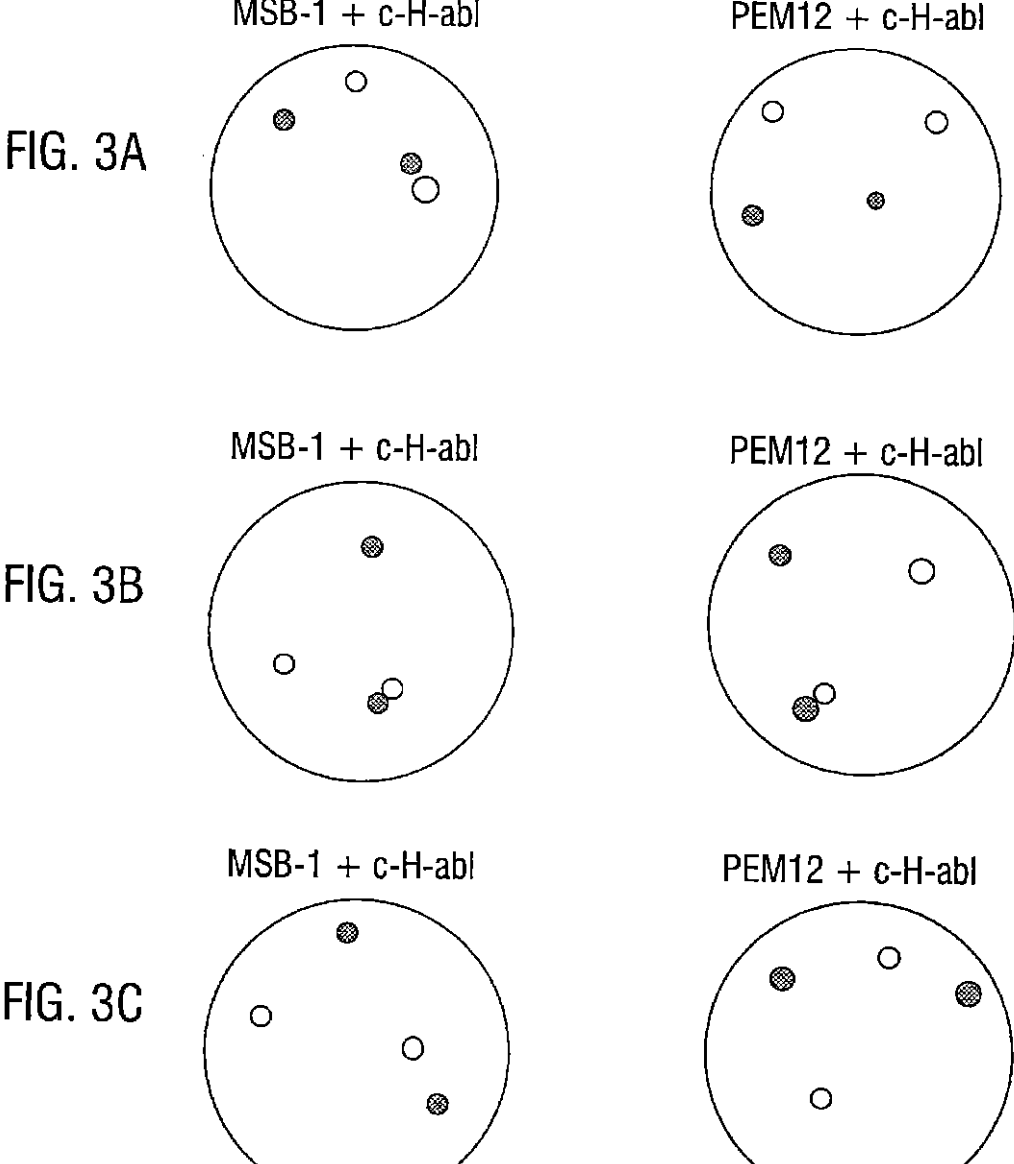

FIG. 3A

FIG. 3B

FIG. 3C

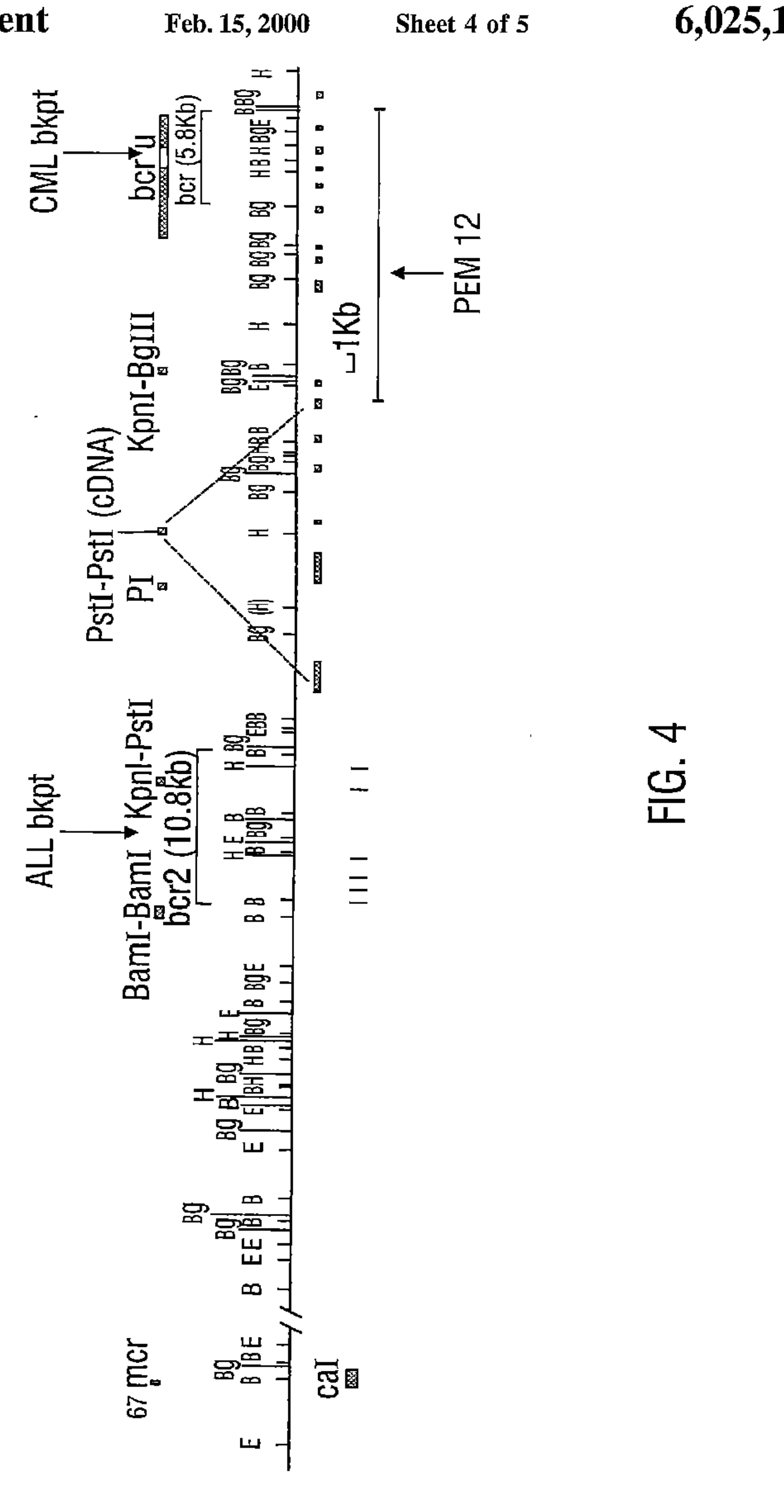

FIG. 4

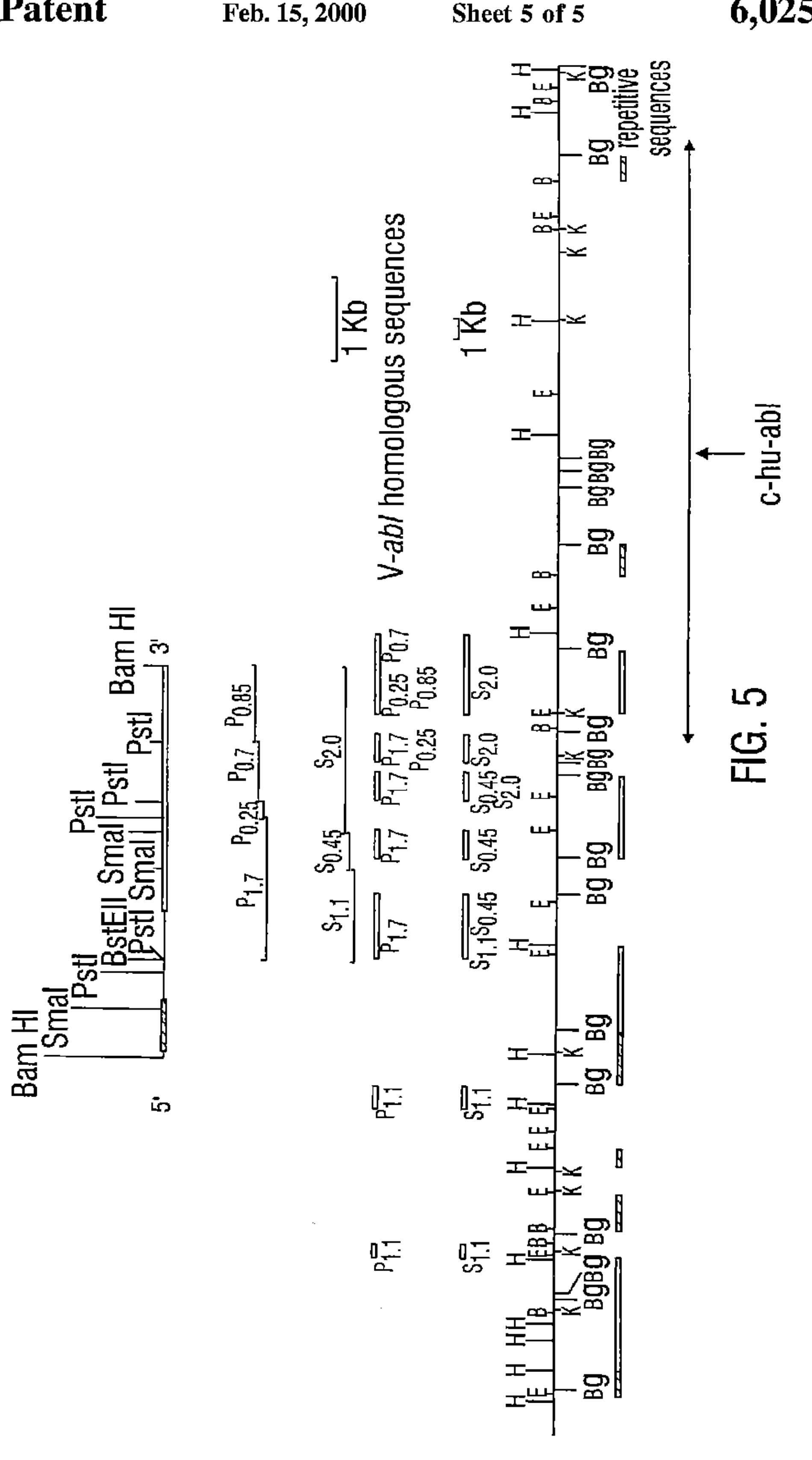

FIG. 5

METHODS AND COMPOSITIONS FOR THE DETECTION OF CHROMOSOMAL ABERRATIONS

The government may have certain rights in this invention persuant to research funding provided by the National Institutes of Health, NIH R29-CA44700.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to methods and compositions for direct detection of specific nucleic acid sequences associated with flanking regions of chromosomal aberration breakpoints, by forming hybrids between the sequences and genetic probes, and detecting the probes. In particular aspects, the invention concerns detection of nucleic acid sequences in situ in chromosomes, and more specifically in cells, including interphase cells. Compositions of probes useful for detecting chromosomal translocations, in particular those associated with human leukemias, are also disclosed.

2. Description of the Related Art

Substantial proportions of human diseases and malformations trace their etiology, at least in part, to genetic factors. Some of these factors are present in the zygote, others occur later as somatic cells form. Detection of genetic factors associated with particular diseases or malformations provides a means for diagnosis and treatment. For some conditions, early detection may allow prevention or amelioration of the devastating courses of diseases.

One class of genetic factors are chromosomal aberrations, that is, deviations in the expected numbers and structure of chromosomes for a particular species, and for particular cell types within a species. These may be constitutive i.e. present in the zygote, or induced post-zygotically in somatic (non-germinal cells) leading to mosaicism, that is a condition where both normal and abnormal cells are present. Chromosomes are the microscopically visible entities that are composed of the genetic material and, in higher organisms such as man, proteins and RNA. The study of chromosomes is called "cytogenetics".

There are several classes of structural aberrations that may involve autosomes or sex chromosomes or both. These aberrations are detected by noting changes in chromosome morphology (band patterns). The band patterns may be only changed in one chromosome (intrachromosomal) or in more than one chromosome (interchromosomal). Normal phenotypes may be associated with these rearrangements if the amount of genetic material has not been altered, but physical or mental anomalies are expected if there is gain or loss of genetic material. Simple deletions (deficiencies) refer to loss of part of a chromosome. Duplication refers to addition of material to chromosomes. Duplication and deficiency of genetic material can be produced by simple breakage of chromosomes, by errors during DNA synthesis, or as a consequence of segregation of other rearrangements into gametes.

Translocations are interchromosomal rearrangements effected by breakage and transfer of part of chromosomes to different locations. In reciprocal translocations, pieces of chromosomes are exchanged between two or more chromosomes. Generally, the exchanges of interest are between nonhomologues. If all the original genetic material appears to be preserved, this condition is referred to as balanced. Unbalanced forms have duplications or deficiencies of genetic material associated with the exchange; that is, something has been gained or lost "in the shuffle."

One of the most exciting associations between chromosomal aberrations and human disease, is that between chromosomal aberrations and cancer. These aberrations are generally not constitutive, i.e., present in the zygote, therefore are not present in all cells—only the abnormal ones. A mosaic condition is said to exist. For example, the Philadelphia (Ph^1) chromosome is an important cytogenetic finding in chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). This chromosome was originally identified as a chromosome sized slightly smaller than a "G-group" chromosome. It was believed to be a deleted chromosome until detection of a reciprocal translocation between chromosomes No. 9 and 22 was reported by Rowley. A reciprocal translocation is one caused by breakage of at least two chromosomes and reunion of the broken piece in new locations.(FIG.1) This aberration was first found to be associated with CML, but is now known to have prognostic and diagnostic value for many hematopoietic malignancies, e.g. ALL.

It is not only the translocation per se that is of clinical interest, but rather the resulting fusion of the proto-oncogene abl from the long arm of chromosome 9 with the bcr gene of chromosome 22, a consistent finding in CML. This genetic change leads to formation of a bcr-abl transcript that is translated to form a 210 kD protein present in virtually all cases of CML. This fusion can be detected by Southern analysis for bcr rearrangements or by in vitro amplification (PCR) of a complementary DNA (CDNA) transcript copied from CML MRNA. In approximately 95% of cases, the fusion gene results from a reciprocal translocation involving chromosomes 9 and 22, producing a cytogenetically distinct small acrocentric chromosome called Ph^1. In the remaining cases the genetic rearrangement is more complex, and the involvement of the bcr and abl regions of chromosomes 9 and 22 may not be apparent during analysis of banded metaphase chromosomes. Southern blots, PCR, and metaphase chromosome banding analysis provide complementary, but incomplete, information on CML. They do not permit a genetic analysis on a cell by cell basis in a format in which the results can be related to cell phenotype as judged by morphology or other markers. Thus, assessment of the distribution of the CML genotype among cells of different lineage and maturity has not been possible.

As an example of the prognostic value of chromosomal aberrations, in adult ALL, the Ph^1 chromosome is present in up to one-third of cases, and is associated with a high relapse rate and short survival. In pediatric ALL it is much less common, but it remains one of the few chromosomal abnormalities that continues to carry a poor prognosis in spite of newer, more intensive approaches to treatment. The accurate detection of the Ph^1 is thus an important part of the diagnostic evaluation of patients with ALL.

Unfortunately, the cytogenetic diagnosis of the Ph^1 chromosome in ALL has been limited. Cytogenetic analysis has a high failure rate in this disease, compared to other acute leukemias or to CML. Fewer than 70% of cases have adequately banded chromosomes at metaphases in most reports. "Banding" is a morphological pattern revealed by treating chromosomes to reveal horizontal stripes which vary in width and staining intensity and are characteristic of specific chromosomal regions. As an alternative to cytogenetic analysis, recently, newer methods of chromosomal in situ hybridization with non-isotopically labelled genetic probes have improved and extended the capabilities of cytogenetics. One of these methods is fluorescence in situ hybridization (FISH). In this method, probes are labelled with fluorescent signals that are detectable, generally by

microscopic viewing of colors. Probes are nucleic acid sequences which bind to matching (homologous) sequences, e.g. on chromosomes. Although based on cytogenetic diagnosis, FISH may be performed on interphase cells as well as on metaphases, and may be applied directly to cells from either the peripheral blood or bone marrow without the need for banded karyotypes. The diagnostic utility of FISH with repetitive, centromeric probes in cases of leukemia has been demonstrated in previous studies.

FISH on interphase cells has proven to be a useful method for diagnosis and clinical management in hematologic diseases. However, much of this experience has concentrated on detecting numerical chromosomal abnormalities (single chromosome loss or gain), making use of chromosome-specific alpha satellite probes, which are highly-repetitive, unique sequences that occur within or near the centromere of chromosomes. The centromere is a constriction most readily visible at metaphase of cell division, which occurs at a characteristic location on each chromosome. The development of competitive hybridization methods to eliminate the signal from Alu-type repeats, and improvements in optics and reagents, have also made it possible to visualize single-copy genomic clones by FISH. However, the use of genomic clones is more difficult than the use of alpha satellite probes, because of lower signal intensity and high background. These difficulties would be offset if use of genomic clones produced improvements in disease assay specificity and were more flexible. Genomic clones are those that contain repeated sequences and non-coding sequences, that is DNA as it exists in the chromosome.

Some of the background for the present invention is as follows: single stranded synthetic DNA was developed with multiple sites are incorporated where fragments may be used as probes. (Stephensen, U.S. Pat. No. 4,681,840). Oncogenes are genes whose products have the ability to transform eukaryotic cells so that they grow in a manner analogous to tumor cells. Probes and methods for detecting chromosomal translocations are disclosed in EPO 181 635 (Groffen et al.)

Pinkel et al. (1986, 1988) and Gray et al. (1990) relate fluorescent-labeled probes for the cytogenetic analysis of chromosomes, and in situ hybridization of chromosomes at metaphase and interphase with whole chromosome-specific DNA.

In situ hybridization using a mixture of radioactive labelled probes c-abl and bcr sequences were employed on a CML patient sample. Although a translocation was said to be detected, Poisson analysis, a statistical procedure, was required to differentiate random from non-random silver grain distribution after autoradiography. (Bartram et al., 1987).

Benn et al. (1987) relates the molecular genetic analysis of the bcr rearrangement in the diagnosis of CML. Analysis involved Southern blots and radioactively labelled probes.

A single bcr-derived probe from which highly repetitive sequences were removed, was employed to detect the Ph^1 translocation in CML. Restriction fragment length polymorphisms (RFLP) were used to identified patients affected with CML. Probes were used to map the chromosome 22 breakpoints within the bcr region by Grossman et al. (1989). Two separate bcr-specific probes were used to detect rearrangements within the bcr region. Southern blots and RFLP were employed. (Hutchins et al., 1989).

Flow cytometry has been applied to detection and characterization of disease—linked chromosome aberrations (Gray et al., 1990). There is a great need to improve methods of detecting specific chromosome aberrations. Flow cytometric requires in vitro cell culture, expensive equipment, and expertise in interpretation of statistical analyses of results. Therefore, it is not generally clinically useful.

Detection of aberrations by use of repeat sequence probes found near centromeres, generally alpha satellite probes, or whole chromosome probes not probes specific for genetic regions associated with diseases. Greater sensitivity and increased resolution is needed. Use of whole chromosome probes is generally limited to detection of aberrations that occur homogeneously in a cell population (Gray et al., 1990) and does not have the resolution to distinguish similar, but distinct breakpoints. The present invention relates methods and compositions for detection of chromosomal aberrations that need not be present in all cells of a sample. Compositions include novel probes that were specifically designed to detect the BCR-ABL fusion gene in acute and chronic leukemias e.g. CML and ALL, and to determine molecular subtypes.

Methods using a plurality of probes to provide increased sensitivity and specificity in detecting chromosomal aberrations, are also aspects of the present invention. These methods are particularly valuable in being applicable to interphase cells, thus avoiding the costly, laborious, time-consuming and often inconclusive cytogenetic analysis of metaphase chromosomes, and the expertise needed for flow cytometry. Not only are the methods of the present invention easier to use, but these methods do not require invasive or risky techniques inflicted on patients, such as bone marrow sampling. However, the methods and compositions of the present invention may also be used on metaphase chromosomes or Souther blots.

SUMMARY

Substantial proportions of human diseases and malformations trace their etiology, at least in part, to genetic factors. Some are inherited, some occur during the development and life of the organism. Cancers, for example, are associated with somatic mutations and/or chromosomal aberrations that may be specific for cancerous cells. Detection of genetic factors associated with particular diseases or malformations provides a means for diagnosis and treatment. For some conditions, early detection may allow prevention or amelioration of the devastating courses of diseases. For others, monitoring the course of the disease is useful to determine treatment strategies. The methods and compositions of the present invention provide multipronged reconnaissance into the genetic material to determine if it harbors abnormal factors.

This invention concerns genetic factors in the form of chromosomal aberrations, that is, deviations from the number and structure of chromosomes characterizing a species, and cell types within the species. In humans, for example, there are generally 46 chromosomes in somatic, i.e. non-germinal cells. These exist in 23 pairs, 22 of which are each matched by size and structural morphology. Structural morphology is revealed by a variety of methods, for example, treatment of chromosomes to form distinguishable horizontal bands. Analysis of such patterns, and comparison of the relative size of the chromosomes and positions of the centromere, a constriction visible at metaphase of the mitotic cell cycle on each chromosome, allow identification and classification of each pair. Analysis of banding patterns also permits detection of structural aberrations both between and within chromosomes.

For purposes of the present invention, structural chromosomal aberrations which comprise a breakpoint fusion

region with nucleic acid sequences flanking the breakpoint fusion, are of particular interest. Flanking regions should be within 800 kb or less so that there are include a particular breakpoint, yet are far enough on either side of the fusion so that they are not included in it. An aspect of the present invention is to detection aberrations which are not detectable by conventional metaphase cytogenetics using light microscopy.

Chromosomes contain linear sequences of DNA, a nucleic acid that is the genetic determinative for most species (RNA is the genetic material in some lower organisms). cloning technology has been developed which is capable of isolating specific genes directly from the genome.

To identify specific genes, that is, specific nucleic acid sequences, specific probes may be used that react only with the particular sequence of interest to seek it out from the vast excess of other sequences. The reaction of probes and their matching (homologous) sequences, is termed hybridization—the joining of the probe and its match by hydrogen bonds. Laboratory methods related to cloning technology and other techniques well known to those of skill in the art, may be found in Maniatis (1982) and in Lewin (1987). Conditions of varying stringency are used depending on the degree of homology required for a match. In examples disclosed herein, stringency conditions are set forth that are specific for hybridization to unique breakpoint provided in preferred embodiments.

The power of this approach in cytogenetic analysis comes from the increasing availability of chromosome- or locus-specific-nucleic acid probes. These fall into three general classes: 1) probes for sequences that are present in many copies on one chromosome, 2) composite probes composed of many individual elements that are homologous to target sequences distributed more-or-less continuously along an entire chromosome, and 3) probes homologous to a specific chromosome subregion or locus; for example, associated with a genetic disease. To use probes to detect chromosomal aberrations formed by breakage and reunion of, e.g., two chromosomes from different pairs (non-homologous), by probing the sequence at the fusion of the breakpoints themselves, may provide a weak signal by which the hybridization is detected. This is because when a short sequence hybridizes, the signal may be too weak to be detected. If a probe is lengthened to provide greater signal intensity, it may become too large. This will be seen as a diffuse signal. Those of skill in the art will readily determine optimum probe size for a particular application using the guidelines disclosed herein.

A laboratory procedure that produces increased specificity and low background, and one in which individual probes can be distinguished as separate entities, is preferred. Thus, the labelling of probes is not amenable to current radioactive isotopic labels, but is more suitably performed with fluorescent and other non-isotopic, i.e. enzymatic or chemical labeling methods. For diagnosis using interphase cells, that stage of cell division that most somatic cells sampled clinically are in, these labelling methods provide good enough intensity to be detectable.

An upper limit on probe size for purposes of the present invention is believed to be about 200 kb of nucleic acids, that is, about 3 times the size used in the examples disclosed herein. A goal in determining suitable sizes for probes is to detect doublets. Doublets are pairs of distinct probes in closer proximity than expected based on there normal chromosome locations in the absence of aberrations. To overcome limitations inherent in some other techniques, this invention provides a strategy of multiple sorties into the genetic material using at least two probes for separate, but related sequences; for example, one for each of the flanking regions of a breakpoint at which fusion of two chromosomal segments has occurred. Moreover, this invention takes advantage of probes large enough to give an intense signal yet specifically targeted to a genomic sequence. To be distinguishable yet juxtaposed at interphase, labelled flanking regions have to be approximately within 800 kb.

The probes are preferably labelled so that their location in the genetic material may be determined. The location is generally determined by use of a microscope. To avoid increased time and the usual problems and risks associated with radioactive labels, fluorescent labels are preferred. A separate color for each independent probe provides the most information. e.g. red on one probe, green on the other.

The probes may be detected in situ, that is, without extraction of the genetic material. In general, it will be the cell, or, more specifically, the cell nucleus that will be viewed.

Although the cells may be analyzed in metaphase, a stage in cell division wherein the chromosomes are individually distinguishable due to contraction, the methods and compositions of the present invention are particularly useful for interphase, a stage in cell division wherein chromosomes are so elongated that they are entwined as is a bowl of spaghetti, and cannot be individually distinguished. At this stage the chromosomes may be referred to as chromatin.

An additional aspect of the invention is the use of genomic DNA fragments as probes, rather than fragments which correspond simply to transcribed/translated regions. By employing genomic fragments of up to 100 kb, e.g., through the use of cosmid clones, it is possible to obtain much greater relative degree of hybridization with the chromosomal DNA, a particular advantage where a light-microscopic detection is envisioned such as in the preferred method in the present invention.

Using multiple probes, each with a distinguishable label, the overall pattern of the probes is used to assay for a breakpoint. Because allelic genes exist on chromosome pairs, each labelled probe capable of hybridizing to a sequence normally present on a specific chromosome, appear twice. If one member of each of two chromosomes is involved in a translocation which moves the sequences hybridizing to the probes together on one fusion chromosome, two of the different colored probes will be in closer proximity to each other than expected if they maintain their original chromosome location, the other two will be more distant.

In particular aspects of this invention, specific disease entities are analyzed. The hematological malignancies provide illustrative embodiments as disclosed in the following sections. One of the most clinically useful assays for chromosomal aberrations is cytogenetic analysis directed at detection of the Philadelphia chromosome (Ph^1) which is associated with chronic myelogenous leukemia (CML), and other hematological malignancies. The presence or absence of the Ph^1 chromosome is a major diagnostic and prognostic aid. However, detection by cytogenetic analysis and other available techniques is time consuming, laborious, and not completely accurate.

An aspect of the present invention concerns the use of DNA probes for the direct detection of Philadelphia chromosomes in metaphase and interphase cells using non-radioactive methods. The so-called Philadelphia chromosome is a chromosomal aberration which results from a

translocation between chromosome 9 and 22 which produces a longer chromosome 9 and shorter chromosome 22. The shortened chromosome 22, termed Ph^1, is generally diagnostic of certain types of leukemia, including in particular chronic myelogenous leukemia (CML), as well as various other leukemias. On a molecular level, it has been shown that the development of the Ph^1 chromosome includes a translocation of a portion of the c-abl oncogene into a breakpoint cluster region (bcr) of chromosome 22, which can activate the ABL gene.

A variety of molecular methods are known for diagnosing this abnormality in DNA or RNA extracted from cancer cells. The method of the present invention involves the use of a specific set of DNA probes, some corresponding to the abl gene, and some corresponding to the bcr gene. This specific set of probes is hybridized in situ to fixed cells of a sample from an individual suspected of being affected. The ABL and BCR specific probes are preferably labeled with separate fluorescein tags (e.g., biotin plus fluorescein-labeled avidin, or digoxigenin-labeled probes). Therefore, upon hybridization, both sets of labeled probes will hybridize to an a translocated chromosome 9 producing a two color doublet, whereas only the ABL specific probe will hybridize to chromosome 9 in non-affected individuals. The use of a visually detectable label allows a means of assessing the presence of the Ph^1 chromosome through the application of light microscopy, providing a significant advantage in terms of expertise required to carry out the assay. The methods are simpler and more rapid than previously available.

Probes developed as an aspect of the present invention include three probes that are particularly useful for detection of hematopoietic malignancies, notably chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). The novel probes are designated: PEM12, c-H-abl and MSB-1. These probes are specific for regions of the BCR gene (MSB-1 and PEM12) and a region at the ABL gene (c-H-abl). The BCR and ABL regions are those which flank the breakpoint fusion region in the Ph^1 chromosome associated with leukemias.

FISH was used to detect the Ph^1 chromosome or its genetic equivalent as the fusion of BCR and ABL probes labeled with two colors. The method was successfully used in interphase cells of ALL patients. This method, using only two probes, only detects the p210 subtype of BCR-ABL gene fusions, whereas the majority of Ph^1-chromosome-positive ALL cases contain the p190 fusion. For this reason, a combination of three probes used in pairs was developed that could detect both the p190 and p210 molecular subtypes. Methods using these combined probes are useful for Ph^1 chromosome detection by FISH in ALL. Capabilities and limitations of probes and combinations of probes in the clinical setting were assessed and shown to provide improvements over previous assays for leukemias, in particular cytogenetic analysis of metaphase chromosomes. Although the embodiments herein relate to detection of chromosomal aberrations in leukemias, the probes may be specifically tailored to meet clinical needs for the diagnosis of any chromosomal aberration, as they have for translocations in leukemias. It is only necessary to be able to determine breakpoint regions and to develop probes to those regions.

The Philadelphia (Ph^1) chromosome is also an important prognostic indicator in acute lymphocytic leukemia (ALL). Present in 30% of adult and 5% of pediatric cases, its presence portends a short remission duration and poor survival, despite improvements in therapy as in CML. It is a derivative of a translocation between chromosomes 9 and

22, and results in the fusion of a part of the ABL proto-oncogene on 9q with part of the BCR gene on chromosome 22. Molecular analysis shows it is much more heterogeneous because the BCR breakpoints are variable. Cytogenetic diagnosis of the Ph^1 chromosome in ALL is possible in only 70% of cases because of the failure to obtain adequate metaphases. The new technique of fluorescence in situ hybridization (FISH) offers the advantage of allowing the diagnoses of chromosomal abnormalities in interphase cells, thus overcoming the problem of metaphase preparations.

Using dual-color FISH with probe combinations specifically tailored to flank the breakpoints in the two types of the BCR-ABL fusion genes p210 and p190, the presence or absence of the Ph^1 chromosome in interphase cells was determined from 5 ALL patients, two ALL-derived cell lines, and normal lymphocytes and specified its molecular subtype when present. The method proved accurate for detection in all cases and for subtyping in 7 of 8 of the cases examined. The sensitivity and specificity for assessing the Ph^1 status of individual cells were low, but results were unequivocal when several cells were examined in a sample.

As can be seen from the following descriptions and examples, the methods disclosed may be performed by a pathologist on routine examination of blood and tissue samples.

FIGURES

FIG. 1 is a schematic representation of a reciprocal translocation between chromosomes No. 9 and 22.

FIG. 2A, FIG. 2B and FIG. 2C is a schematic representation of the location of probes used for dual-color FISH on the normal BCR gene (FIG. 2A) and on BCR-ABL fusion gene subtypes (FIG. 2B—The p210 fusion gene; FIG. 2C—The p190 fusion gene).

FIG. 3A, FIG. 3B and FIG. 3C illustrates expected signal (labelling) patterns associated with different molecular subtypes of the Ph^1 in interphase cells using two-color FISH with two different probe combinations used (FIG. 3A—p190 p fusion gene; FIG. 3B—p210 type fusion gene; FIG. 3C—normal or negative).

FIG. 4 is a restriction enzyme map of part of the human BCR gene from chromosomal 22.

FIG. 5 is a restriction enzyme map of the human c-abl region from chromosome 9.

DETAILED DESCRIPTION OF THE INVENTION

The following examples, materials and methods provide embodiments of the invention.

EXAMPLE 1

Detection of Ph^1 in CML

Two-color FISH with the abl (red) or bcr (green) probe to normal G_1 interphase nuclei in most cases resulted in two red and two green hybridization signals that were well separated and randomly distributed around the nucleus. In a few cells,, two doublet hybridization signals were detected, probably as a result of hybridization to both sister chromatids of both homologs in cells that had replicated this region of DNA (those in the S or G_1 phase of the cell cycle).

Depending on the exact positioning of the breakpoints in the leukemic clone, the genetic rearrangement of CML brings the binding sites of the bcr and abl probes to within 25 to 225 kb of each other on an abnormal chromosome.

Dual-color hybridization with abl and bcr probes to interphase CML cells resulted in one red and one green hybridization signal located randomly in the nucleus, and one red-green doublet signal in which the separation between the two colors was <1 μm. In some cases, the red-green doublet appeared yellow. The randomly located red and green signals are likely due to hybridization to the abl and bcr genes on the normal chromosomes, and the red-green doublet signal to hybridization to the bcr-abl fusion gene. The distance between the red and green components of the fusion signal is consistent with interphase mapping studies. Those studies have shown that DNA sequences separated by less than 250 kb should be within 1 μm of each other in two-dimensional interphase nuclei. Since the positions of the bcr and abl hybridization sites are distributed apparently randomly over the two-dimensional nucleus images in normal cells, it is not surprising that some normal cells will have red and green signals separated by <1 μm. Such false positive cells were found at a frequency of about 1% (9 of 750 cells pooled from four normal individuals). The highest frequency of false positive fusion signals for an individual case was 3 of 150 cells analyzed. Thus, with the use of this probe placement strategy, these results set a practical limit of about 1% for the detectable frequency of CML cells in a population.

Hybridization results for seven samples from six CML cases and data from PCR, Southern, and chromosome banding analysis are presented in Table 1. In all six cases red-green hybridization signals separated by <1 μm in more than 50% of nuclei were present. This was the case in three cases found to be Ph[1]-negative by banding analysis (CML-4, CML-5, and CML-6). In most cases, the fusion event was visible in virtually every cell. One case (CML-6) showed fusion signals in almost every cell despite the fact that PCR analysis failed to detect the presence of a fusion mRNA and banding analysis did not reveal a Ph[1]. Hybridization to metaphase cells was performed in three cases (CML-1, CML-4, and CML-5). Red and green hybridization signals in close proximity on a single small acrocentric chromosome were present in all three. In two cases (CML-1 and CML-4) scored as t(9:22) (q34:q11) by banding analysis, the red-green pair was in close proximity to the telomere of the long arm of a small acrocentric chromosome is expected for the Ph[1].

From the results of banding analysis one case (CML-5) was suspected to have an insertion of chromosomal material at 22q11. Two-color hybridization to metaphase cells from this case showed the red-green pair to be centrally located in a small chromosome. This result is consistent with formation of the bcr-abl fusion gene by an interstitial insertion. Fusion genes are not always detectable by cytogenetic banding analysis of metaphase chromosomes.

In one case (CML-1), two pairs of red-green doublet signals were seen in 3 of 150 (2%) interphase nuclei. This may indicate a double Ph[1] (or double fusion gene) in those cells that was not detected by banding analysis, which was limited to 25 metaphase cells. The acquisition of an additional Ph[1] is the most frequent cytogenetic event accompanying blast transformation, and its cytogenetic detection may herald disease acceleration.

Samples CML-3a and CML-3b represent an analysis of peripheral blood and bone marrow, respectively, from the same patient. The percentage of bcr-abl fusion-positive cells was higher in the bone marrow than peripheral blood.

TABLE 1

A summary of cytogenetic, FISH, and other analyses of bcr-abl rearrangements in six CML cases. CML-1 and CML-5 were bone marrow samples from patients with chronic phase CML who were receiving no treatment. CML-3a and CML-3b were from peripheral blood and bone marrow, respectively, of a CML patient in blast crisis, who was receiving hydroxyurea, CML-2 was from bone marrow in a blast crisis CML patient. CML-4 was bone marrow from a CML patient in blast crisis and receiving no treatment. CML-6 was from bone marrow in a chronic phase CML patient receiving hydroxyurea. Hybridization to metaphase cells was done on cases CML-1, CML-4, and CML-5. CML-1 and CML-4 both showed fusion gene signals localized near the end of a small acrocentric chromosome consistent with a classic Ph[1] resulting from a reciprocal translocation. CML-5 showed an interstitial fusion signal on 22q consistent with the bcr-abl fusion gene resulting from an insertional event. F, fusion; N, normal; D, double fusion; NI, not interpretable; and ND, not done.

Patient	Cytogenetics	Interphase FISH	Other Analysis
CML-1	46,XX,t(9;22)(q34;q11)	80% F 2% D 18% NI	ND
CML-2	46,XY,t(9;22)(q34;q11)	60% F 40% NI	PCR[b]
CML-3a	46,XY,t(9;22)(q34;q11)	75% F 25% N	PCR[a]
CML-3b	46,XY,t(9;22)(q34;q11)	100% F	PCR[a]
CML-4	47,XY, + 8,del(22)(q11)	100% F	PCR[b]
CML-5	46,XY,ins(22;9)(q11;q34q34)	100% F	PCR[b]
CML-6	46,XY,t(5;9)(q13;q34)	100% F	PCR[b] Southern[c]

[a]See Materials and Methods, PCR, Kohler.
[b]See Materials and Methods, PCR, Hogewisch
[c]See Materials and Methods, Southern Blot.

EXAMPLE 2

Detection of Ph[1] in ALL

Location of probes used for dual-color FISH on the normal BCR gene and on BCR-ABL fusion gene subtypes is shown in FIG. 2A, FIG. 2B and FIG. 2C. The normal BCR gene, and the two subtypes of BCR-ABL fusion gene are schematically represented. Black vertical bars represent BCR exons, with the first exon indicated by Roman numeral I, and the breakpoint cluster region indicated by "bcr." Open vertical bars represent ABL exons, and the second exon is indicated by Roman numeral II. The diagrams are not to scale; the approximate total distance of the BCR gene is 130 kb; the total extent of the ABL regions depicted here is 40 kb.

FIG. 2A. The normal BCR gene showing the approximate location of the MSB-1 probe, and the PEM12 probes (not to scale).

FIG. 2B. The p210 subtype of the BCR-ABL fusion gene as found on the Ph[1] chromosome, showing the approximate location of the MSB-1 probe and the PEM12 probe, relative to the c-H-ABL probe. The jagged line indicates a possible translocation breakpoint.

FIG. 2C. The p190 subtype of BCR-ABL fusion gene, with a representative breakpoint indicated by the jagged line and the approximate locations of the MSB-1 and c-H-abl probes in this gene. The breakpoint is located proximal to the PEM12 probe, and it there-

fore translocates to the 9q+ chromosome and is separated from the ABL sequences.

Detection of the Ph[1] in interphase cells using two-color FISH with probe combinations used and expected signal patterns given by different molecular subtypes is shown in FIG. 3A, FIG. 3A, FIG. 3B and FIG. 3C. Open circles represent the green signal of fluorescein-conjugated anti-digoxigenin antibodies to detect the MSB-1 or PEM12 probes and hatched circles represent the red signal of avidin—Texas red used to detect the biotinylated probe c-H-abl.

FIG. 3A. The p190 fusion gene will show a pattern of doublets with the MSB-1+c-H-abl combination but result in separated doublets with PEM12+c-H-abl.

FIG. 3B. The p210 fusion gene show doublets with both probe combinations.

FIG. 3C. Normal or Ph[1]-negative ALL cells show separated signals with both probe combinations.

FIG. 4: illustrates a restriction enzyme map of part of the human BCR gene from chromosomal 22. B=BAM H1; Bg=Bg111; E=EcoR 1; H=Hin d111. The dark area in brackets below the map indicates the PEM12 region, an approximately 18 kb insert in lambda phage. Arrows indicate breakpoints in ALL and CML.

FIG. 5: illustrates a restriction enzyme map of the human c-abl region from chromosome 9. The arrow below the map indicates the c-hu-abl cosmid region, approximately a 30 kb insert.

Detection of Fusion Gene Subtypes

The detection of the two BCR-ABL fusion gene subtypes is outlined in FIG. 3A, FIG. 3B and FIG. 3C. The molecular basis of the Ph[1] chromosome is a translocation between the long arms of chromosomes 9 and 22, t(9;22)(q11;q34) (Rowley, 1973) (FIG. 1 where the hatched chromosome material was originally on chromosome 9, the clear on chromosome 22; after breakage and reunion, two derivative chromosomes are formed, the small being the Ph[1] chromosome which juxtaposes part of the ABL protooncogene on chromosome 9q34 (Kurzrock et al., 1988) next to part of the BCR gene on chromosome 22q11.) The resulting fusion gene is transcribed and translated to produce a chimeric protein. Two types of BCR-ABL fusion genes exist in ALL. One type has a BCR breakpoint in the limited region of the M-bcr (Groffen et al., 1984) and produces a 210 kd protein, designated p210. This is the type of fusion gene found in virtually all cases of CML. The other type of fusion gene has a BCR breakpoint in the large region of the BCR first intron (Rubin et al., 1988; Heisterkamp et al., 1988) and it produces a 190 kd protein, p190. This type of fusion gene accounts for 75% of the Ph[1] positive cases of ALL, the remainder having the p210 rearrangement.

A map of the normal BCR gene (Hooberman et al., 1989) and the two types of fusion genes, with probe localizations, is shown in FIG. 2A, FIG. 2B and FIG. 2C. The c-H-abl probe contains the last exon of the ABL gene, which is a necessary part of the BCR-ABL fusion gene. The MSB-1 probe contains the first exon of the BCR gene, while PEM12 lies immediately 5' of the M-bcr. Both the PEM12+c-H-abl and MSB-1+c-H-abl probe combinations produce doublets when a p210 type of fusion gene is present,, because both of these regions of the BCR gene are retained on the Ph[1] chromosome. When a p190 fusion gene is present, however, the breakpoint exists between these two probes, so that only MSB-1 is retained on the Ph[1] chromosome to fuse with c-H-abl, while PEM12 remains on the 9q+chromosome. Consequently, doublets are seen with MSB-1+c-H-abl but not: with PEM12+c-H-abl.

In FIG. 2A, the regions of the BCR gene to which the two probes, MSB-1 and PEM12 hybridize, are shown. A labelled MSB-1 probe contacted to chromosomes which hybridized to a normal human chromosome No. 9, would be detected in association with that chromosome. Similarly, a labelled P:E:M12 probe would hybridize with a chromosome No. 9 and show association with it by detection of the label.

The nucleic acid sequence of the ABL gene is normally on chromosome No. 22. The probe c-H-abl will hybridize to a region of the ABL gene as shown in FIG. 2B. Also shown in FIG. 2B are the locations of the p210 subtype of the fusion gene formed after breakage and reunion of chromosomes No. 9 and 22 to form a fusion gene. This fusion gene is designated p210 because it is capable of being expressed as a fusion protein with a molecular weight of approximately 210 kd as determined by SDS gel electrophoresis.

Application of both probe combinations thus permits the both the detection of the BCR-ABL fusion gene, and specification of the subtype. A schematic diagram of expected results in interphase is shown in FIG. 3A, FIG. 3B and FIG. 3C. Because the ABL breakpoint is variable, the probe separations may be from 25 to over 200 kb. In metaphase in situ hybridization, this range of distances will cause the probe signal to fuse, but in interphase in situ hybridization the two colors can be resolved.

If combinations of two dual labelled probes are added to a preparation containing the p210 subtype of fusion gene, a pattern in interphase cell nuclei will appear as showing a dual labelled doublet where the fusion gene is located, and two single labels where the normal (untranslocated) chromosome Nos. 9 and 22 are located (FIG. 3B). In FIG. 3B, the appearance of doublets of MSB-1+c-H-abl, and PEM12+c-H-abl, are shown schematically.

The presence of the p190 subtype of fusion gene can be distinguished from the presence of the p210 fusion gene, or the absence of fusion genes of this type in normal cells (FIG. 3), because the p190 fusion gene does not have a PEM12 site (FIG. 2C). Therefore, as shown in FIG. 3A, labelled doublets reflecting probe associations are only expected with the combination of probes MSB-1+c-H-abl. Contacting cells with the probe combination PEM12+c-H-abl will not yield doublets, and should produce an appearance at interphase of a cell pattern undistinguishable from normal cells. (Compare FIGS. 2A and 2C).

Five cases of newly-diagnosed adult ALL, were analyzed using the methods of the present invention. Results are shown in Table 2. Cases 1 to 3 were peripheral blood samples, but all had greater than 50% blasts in the sample; cases 4 and 5 were bone marrow samples. Determination of the presence or absence of a fusion gene agreed with the molecular results in each case. The two negative cases were easily scored. Among the BCR-ABL positive cases, the subtype was obvious in patients 3 and 5, but indeterminate in patient 2. For patient 2, the MSB-1+c-H-abl determination was unequivocal, and many cells even contained two doublets; however, the result for the PEM12+c-H-abl combination was indeterminate, and no cells were observed which contained multiple doublets. Several subsequent attempts to repeat the assay with the PEM12+c-H-abl combination failed to resolve the issue.

Variability between runs was apparent. The best hybridization results allowed the interpretation of one cell in three. Any hybridization in which no more than one cell in 8–10 showed results was discarded. Factors which affected the quality of hybridization results included quality of the sample, with best results from freshly fixed (<1 day in fixative) samples showing even, rather than clumped, chromatin condensation, and minimal residual cytoplasm. Other factors included the hybridization efficiency of the probes, and the ability to visualize the doublets, which were sometimes difficult to resolve into two different colors. The final problem was greatly improved by a change from fluorescein-tagged anti-digoxegenin Fab fragments to new, polyclonal, whole-antibody anti-digoxigenin antibodies (both by Boehringer Mannheim). Of the three probes, the c-H-abl cosmid produced the most evaluable cells, followed by PEM12, with MSB-1 as the least efficient of the probes. Of hybridizations which were discarded for failure of one probe to hybridize, the most often to fail was MSB-1.

The presence or absence of the BCR-ABL fusion gene were correctly assigned in 2 cell lines and 5 clinical specimens of ALL. The molecular subtype was easily specified for most of these cases except for case 2, in which the relatively high false positive rate (17%) made interpretation difficult.

The analysis was performed directly on interphase is specimens of peripheral blood or bone marrow, obviating the need for cell culture and metaphase preparation. The ability to detect chromosomal abnormalities in interphase cells has an important impact on ALL, because failure to obtain adequate metaphases is one of the most common reasons for cytogenetic failure in this disease. Another important feature of FISH compared to conventional cytogenetics is that it directly detected the important molecular events—the BCR-ABL gene fusion subtype—rather than merely the presence of chromosomal aberration. In this regard, it compares favorably to molecular methods of diagnosis. The BCR-ABL fusion gene is an important clinical finding in ALL, and FISH is a viable option for its detection. The limitations and capabilities of the disclosed methods were evaluated in a clinical setting.

Although the ability to diagnose a case where more than one cell was available for analysis, which will generally be the situation presented, was excellent, the ability to detect the Ph^1 in an individual cell was rather more limited. The rate of detection of a positive signal was lower than might be predicted. In the cell lines, which are believed to contain 100% Ph^1 positive cells, the range of detection was 46–83% positive cells, with an overall average of 68%. In the patient samples, the average number of cells scored as positive was lower; however, these are heterogeneous populations of cells, and lower FISH results for patients relates to the lower percentage of malignant cells in the samples. There will generally be both normal and malignant cells in a sample, and the percentages will vary from patient to patient, and even within samples from the same patient.

Similarly, the rate of detection of a false positive doublet signal is 3 to 10% of cells of normal donors.

In summary, interphase detection of FISH with these probe combinations are an accurate method of detecting the presence of the Ph^1 chromosome in ALL, and may be a technique which will afford this diagnosis in nearly 100% of ALL patients.

EXAMPLE 3

Detection of Ph^1 in a Cell Line

Cell Lines and Normal Lymphocytes

The two probe combinations were tested on preparations of normal lymphocytes, a cell line with a known p210 gene fusion (BV173), and a cell line with a known p190 gene fusion (SUPB13). The results of these determinations, performed blindly, are shown in Table 3. It was found that the presence or absence of either subtype of the BCR-ABL fusion gene could accurately be assessed. However, none of the cell lines gave 100% fusion gene-positive cells, as would be expected from a homogeneous population of cells. The percent of cells scored as positive ranged from 46 to 83% of cells with recognizable signals, indicating a false negative rate of 17 to 56%. The false negatives were highly dependent on degree of background staining, sample quality, hybridization efficiency of the probes, and experience. The rate lowered as the observer became more experienced.

The false positive rate, the number of cells with doublets in normal lymphocytes, or of SUPB13 cells containing PEM12+c-H-abl doublets, ranged from 3 to 10%. Based on this experience, it was found useful to define the positive and negative patient cases relative to normal controls run at the same time. A case was defined as positive when the doublets were present at two-and-a-half times the rate found with negative controls, and as negative if the rate of doublets was similar (within 5%) to the normal lymphocyte results. Any findings in between were scored as indeterminate.

Multiple red-green hybridization sites along both arms of a single acrocentric chromosome were detected in simultaneous hybridization ions with abl and bcr probes to metaphase cells of the CML-derived cell line K-562. Hybridization to interphase nuclei demonstrated that the red and green signals were localized to the same region of the nucleus. This is consistent with their being present on a single chromosome. Eight to 16 hybridization pairs were seen in each of 250 nuclei enumerated, indicating corresponding amplification of the bcr-abl fusion gene. Fusion gene amplification was not seen in any of the normal controls or CML patients analyzed. These findings are consistent with previous Southern blot data showing amplification of the fusion gene in this cell line.

In summary, dual-color FISH analysis of interphase cells from seven CML and four normal cell samples with abl and bar probes suggests the utility of this approach for routine diagnosis and clinical monitoring of CML. A significant advantage of this technique is the ability to obtain genetic information from individual interphase or metaphase cells in less than 24 hours. Its application is not limited to cells that, fortuitously or through culture, happen to be in metaphase; it can be applied to all cells of a population. The genotypic analysis can be associated with cell phenotype, as judged by morphology or other markers, and this makes possible the study of lineage specificity of cells carrying the CML genotype, as well as assessment of the frequency of cells carrying the abnormality. Moreover, counting of hybridization spots allows the determination of the degree of bcr-abl gene amplification in the K-562 cell line. It is possible that this analysis may be further developed using quantitative measurement of fluorescence intensity.

TABLE 2

EVALUATION OF THE Ph[1] CHROMOSOME IN LEUKEMIA SPECIMENS USING DUAL-COLOR FISH.

Case	Patient Data	FISH Probe MSB1 + c-H-ab1	Determinations* (combinations) PEM12 + c-H-ab1	Combined FISH Results	Molecular Findings
1.	16F	92/100 (8%)	14/107 (13%)	Ph[1] negative	BCR-ABL negative
2.	63F	36/114 (31%)**	19/110 (17%)	Ph[1] positive indeterminate	BCR-ABL positive p190 subtype
3.	61M	54/99 (55%)	83/140 (59%)**	Ph[1] positive p210 subtype	BCR-ABL positive p210 subtype
4.	39M	2/59 (3%)	11/118 (9%)	Ph[1] negative	BCR-ABL negative
5.	38M	46/104 (44%)	9/108 (8%)	Ph[1] positive p190 subtype	BCR-ABL positive p190 subtype

*Results are presented as number of cells with doublets/total number of evaluable cells (% cells with doublets)
**Multiple doublets were observed in some cells in these cases.

TABLE 3

EVALUATION OF PROBE COMBINATIONS ON Ph[1] CHROMOSOME-POSITIVE CELL LINES AND NORMAL LYMPHOCYTES.

SAMPLE	MSB-1 + c-H-abl (%)*	PEM12 + c-H-abl (%)*
SUPB13	1. 56/104 (46%) 2. 48/62 (77%) 3. 71/22 (58%)	1. 7/119 (6%) 2. 7/101 (7%)
BV173	1. 80/96 (83%) 2. 76/97 (78%)	1. 43/73 (59%) 2. 80/102 (78%)
Normal Lymphocytes	1. 3/120 (3%) 2. 12/121 (10%)	1. 8/106 (8%) 2. 8/109 (7%)

*Results expressed as number of cells with a doublet/total number of evaluable cells (percent positive cells). Each line represents a separate run.

Materials and Methods

Patient Samples

Cases were selected from newly diagnosed ALL samples referred to the inventor's laboratory for pulsed field gel electrophoresis (PFGE) and Southern molecular analysis for the BCR-ABL fusion gene. Peripheral blood was anticoagulated with EDTA (lavender-top tubes) and bone marrow with heparin (green-top tubes). Buffy coats were removed from the samples and incubated with NH_4Cl (0.135M in 0.005M Tris HCl ph 7.6) to lyse red cells. After several washes in Hank's balanced salt solution (HBSS, Gibco), the cells were counted and viability assessed. Cells for FISH were allowed to "rest" in the final wash solution of HBSS for a few hours at 4° C. Cells for molecular analysis were embedded in agarose plugs according to previously described procedures. (Hooberman et al., 1989). In two cases (Patients 2 and 5), cells for FISH were thawed from liquid nitrogen storage, and incubated without stimulation or colcemid arrest for 3–24 hours prior to harvest.

Two lymphoblastoid cell lines, SUPB13 (Rubin et al., 1988), positive for the p190 type of fusion gene, and BV173 (Westbrook et al., 1988), positive for p210, were used. They were grown in RPMI 1640 (Gibco) with 10% fetal bovine serum and penicillin-streptomycin (Sigma, St. Louis, Mo). Peripheral blood lymphocytes, obtained from normal healthy donors were stimulated with phytohemagglutinin (PHA, 1 mg/ml, Burroughs-Welcome) and cultured for 72 hrs. Cell lines and stimulated lymphocytes were incubated at 37° C. in 5% Co_2. Twenty-five and 5 minutes prior to harvest, Colcemid (Gibco) was added to the culture flasks to a final concentration of 0.1 ug/ml to produce metaphase arrest.

Probes

1. Description

PEM12 is a phage clone containing an 18 kb human genomic insert in EMBL3. It contains part of a sequence of the major breakpoint cluster region (M-bcr) of chromosome 22 and extends 5' of it. M-bcr is an area wherein breakpoints cluster within the BCR gene.

The c-H-abl probe is a cosmid approximately 40 kb in size with a 35 kb human insert in pCV105, and 5 kb of vector, specific for the 3' end of the ABL gene. The cosmid was isolated from a cosmid library 105SL/108K provided by Dr. Chris Y-F Lau. This library is generally available for research use and has been described in *Proc. Natl. Acad. Sci. USA* 80:5225 (1983). Cosmids were hybridized with probes containing contiguous fragments of the genes of interest, here the ABL gene sequences. The procedure was to use probes of increasing size.

MSB-1 is a phage clone with an 18 kb fragment of human DNA from the first exon of the BCR gene cloned into EMBL3.

The PEM12 and MSB-1 probes were labeled with digoxigenin-11-dUTP (Boehringer Mannheim) and the c-H-abl cosmid was labeled with biotin-11-dUTP (ENZO) by nick translation, using reagents supplied by ENZO Diagnostics. The probes were combined with each other in two combinations: MSB-1+c-H-abl and PEM12+c-H-abl. Fifty ng of c-H-abl was combined with 150–170 ng of PEM12 or MSB-1 and 1 μg of human placental DNA. The mixture was brought to a total of 10 μg of DNA/per slide with salmon sperm DNA. The probe combinations were then ethanol precipitated and redissolved in a hybridization solution of 50% formamide/10% dextran sulfate in 2× SSC, heated to 70° C. for 5 min., then incubated at 37° C. for 15–30 min. prior to application to slides.

2. Preparation

The Sequence of the BCR and ABL genes are available in the GeneBank™. The sequences selected for use as probes may be amplified, e.g., by PCR which is well known to those of skill in the art, and used to screen libraries. (Maniatis, 1982).

Ranges of preferred probe sizes and distances from the breakpoint-fusion area, are disclosed in previous sections.

Slide Preparation and Hybridization

Cell lines, normal lymphocytes, or patient cells were pelleted by centrifugation (1000 rpm for 10 min.), and treated with hypotonic KCl (0.075M), for 12 min. at 37° C. They were resuspended in 3:1 methanol:acetic acid for

fixation, and stored at 4° C. until slides were prepared, usually one to ten days. The samples were pelleted again and washed three times in fresh 3:1 methanol:acetic acid fixative immediately prior to dropping onto slides pre-cleaned with 95% alcohol. Slides of the samples were stored desiccated at 4° C. until use.

The slides were baked on a slide warmer for 4 hrs. at 65° C. They were incubated in an RNase solution, 100 μg/ml in 2× SSC for 1 hr. at 37° C., then washed 4 times in 2× SSC, 2 min. each. Next, they were passed through a graded alcohol series (70%, 80%, 95%), 2 min. each and allowed to air dry.

Denaturation of cellular DNA was performed in 70% formamide in 4× SSC for 2 min. at 70° C., and the graded alcohol series and air drying were repeated. A gentle proteinase K digestion, 60 ng/ml in 20 mM Tris/2 mM $CaCl_2$ at 37° C. for 8 min. (Pinkel et al., 1986) was followed by a third graded alcohol series and air drying. The slides were warmed to 37° C. and held there until the probe hybridization mixture was applied. Ten μl of the probe mixture was applied to each slide, the area of hybridization was covered with 22×22 mm coverslip, sealed with rubber cement and placed on a hotplate at 90° C. for 2 min. Two slides from each case were hybridized, one for the MSB-1/c-H-abl, and one for the PEM12/c-H-abl combination.

Detection of Hybridization

Detection steps are essentially those described by Trask et al. (Trask et al., 1991) with minor modifications. Following overnight incubation at 37° C. in a moist chamber, the coverslips were removed and the slides washed 3 times in 50% formamide/4× SSC, 5 min. each at 40° C. To block nonspecific binding, the slides were incubated for 5 min. at room temperature with 100 μl of 3% bovine serum albumin (BSA) (Sigma, St. Louis, Mo.) in 4× SSC under a coverslip.

The first detection reagent, avidin-Texas red (Vector) diluted in 3% BSA/4× SSC (2.5 ug of fluorochrome per ml of diluent) was applied, the coverslip was replaced, and the slides were incubated at 37° C. for 1 hr. The slides were washed 3 times, in 4× SSC, 4× SSC/0.1% Triton X, and PN (0.1M NaH_2PO_4/NaH_2PO_4 buffer pH 8/0.1% NP-40), sequentially, for 5 min. each.

A second blocking step was performed with PMN (PN+5% non-fat dry milk+0.05% sodium azide, centrifuged to remove milk solids), 100 μl was placed under a coverslip for 5 min. at room temperature. The second detection and amplification reagent, anti-digoxigenin polyclonal antibody (Boehringer Mannheim) and biotinylated anti-avidin (Vector), was applied in a 1:25 dilution in PMN (100 ul/slide) and the slides were incubated again at 37° C. for 1 hr. Three washes, 5 min. each, in PN followed. The PMN block step was repeated, and the third fluorescent reagent, avidin-Texas red and fluorescein-conjugated rabbit anti-sheep antibody (Vector) a 1:50 dilution, in PMN, was incubated at 37° C. for 1 hr. The final washes were PN X2, then 4× SSC/0.1% Triton X once for 5 min. A brief (1–2 min.) bath in DAPI (diamidino-2-phenylindole, dihydrochloride; (Sigma, St. Louis, Mo.)) 200 ng/ml in 4× SSC/0.1% TritonX, was followed by a rinse in 4× SSC. The slides were then coverslipped with a DABCO antifade solution (diazabicyclooctane, Sigma) (90% glycerol/2.3% DABCO in 20 mM Tris pH 8.0), and stored desiccated in light-tight boxes at −20° C. until reviewed (usually less than three days later).

Molecular Analysis

Molecular analysis for the presence or absence of the BCR-ABL fusion gene and its subtype was performed by a combination of pulsed field gel and Southern blot, as

described previously (Hooberman et al., 1989). All cases were reviewed by one observer without knowledge of the FISH results.

Interpretation of Slides for Probe Hybridization

The slides were viewed with a Zeiss standard 16 microscope equipped for epifluorescent illumination and a set of dual band-pass filters (Omega Optical). All samples were coded so that the observer did not know the results of the molecular studies at the time of review. At least 100 interphase cells were scored for the presence or absence of a red-green doublet. A doublet was defined as red and green signals lying with a distance of 1 diameter of a signal, approximately 1 micron. Bright yellow signals which could not be resolved into red and green were not counted as doublets. A slide was discarded if failure of hybridization of one or both probes was apparent. When both probe combinations for a case had been scored, an assignment of the presence and subtype of fusion gene was made.

Hybridization of Nucleic Acid Sequences

CML-3b and CML-6: Five to ten drops of marrow diluted with phosphate buffered saline (PBS) to prevent clotting were fixed in methanol/acetic acid and dropped on slides.

CML-1, CML-2, CML-4, and CML-5: Peripheral blood or bone marrow, or both, was cultured in RMPI 1640 supplemented with 10% feral bovine serum, an antibiotic mixture (gentamicin 500 μg/ml), and 1% L-glutamine for 24 hours. Cultures were synchronized according to J. J. Yunio and M. E. Chandler, Prog. Clin. Path. 7, 267 (1977), and chromosome preparations followed L. M. Gibas and Jackson, Karyogrom 9 (1986).

CML-3a: Peripheral blood was centrifuged for 5 min. at 1100 rpm, the buffy coat was pipetted off and diluted with the same volume of PBS, spun down, fixed in methanol/acetic acid, and dropped on slides. Hybridization followed procedures described by Pinkel et al., Proc. Natl. Acad. Sci. U.S.A. 45 9138 (1983), Trask et al., Genomics 6:710 (1989) and Lawrence, Villaive, and Singer, Cell 42, 51 (1988), with modifications.

The bcr probe was nick-translated (Bethesda Research Laboratories Nick-Translation System) with digoxigenin 11-dUTP (deoxyuridine 5'-triphosphate) (Boehringer Mannheim Biochemicals) with an average incorporation of 26%. The abl probe was similarly nick-translated with biotin-11-dUTP (Enzo Diagnostics). Cells were thermally denatured at 72° C. for 5 min., dehydrated in an ethanol series, air-dried, and placed at 37° C. A hybridization mixture (10 μl) containing each probe (2 ng/ul), 50% formamide/2× standard saline citrate (SSC) 10% dextran sulfate, and human genomic DNA (1 mg/ml, sonicated to 200 to 600 bp) was heated to 70° C. for 5 min. incubated for 30 min. at 37° C., placed on the warmed slides, covered with a 20 mm by 20 mm cover slip, sealed with rubber cement, and incubated overnight at 37° C. Slides were washed three times in 50% formamide 2× SSC for 20 min each at 42° C., twice in 2× SSC at 42° C. for 30 min. each, and rinsed at room temperature in 4× SSC. All subsequent steps were performed at room temperature.

Slides were blocked in 100 μl of 4× SSC/1% bovine serum albumin (BSA) for 5 min. under a coverslip. The biotinylated abl probe was detected by applying 100 μl of Texas red-avidin (Vector Laboratories, Inc.), 2 μg/ml in 4× SSC/1% BSA) for 45 min. The slides were washed twice for 5 min. in 4× SSC/1% Triton X-100 (Sigma). The signal was amplified by applying biotinylated goat antibody so avidin (Vector Laboratories, Inc., 5 μg/ml in PNM 0.1M M NaH_2PO_4/0.1 M Na_2HPO_4, pH 8 (PN) containing 5% nonfat dry milk and 0.02% sodium amide and centrifuged to

remove solids), washed twice in PN for 5 min., followed by another layer of Texas red-avidin in PNM. The digoxigenin-labeled bcr probe was detected by incubation with sheep antibody to digoxigenin (obtained from D. Pepper, Boehringer Mannheim Biochemicals, Indianapolis, Ind.; 18.4 μg/ml in PNM) for 30 min, washed twice in PN for 5 min., followed by a rabbit-antibody to sheep conjugated with FITC (Organon Teknika-Cappel, 1:50 in PNM). After washing twice for 5 min. in PN, the signal was amplified by applying a sheep antibody to rabbit immunoglobulin G(IgG) conjugated to FITC (organon Tekniks-Cappel, 1:50 in PNM). The slides were then rinsed in PN. Slides were mounted in 10 μl of fluorescence body solution Johnson and Nogueria, J. Immunol. Methods 43, 349 (1981) containing: 4',6-amidino-2-phenylindols (DAPI) at 1 μg/ml as a counterstain.

The slides were examined with an FITC/Texas red double-band pass filter set (Omega Optical on a Zeiss Axioskap.

PCR Method of Kohler et al.

The method of Kohler et al. [Leukemia 4, 8 (1990)] for bcr-abl PCR on CML-2, CML-4, and CML-5. The oligonucleotide primers used were as follows: ablX3 antisense downstream 5'-TTT CTC CAG ACT GTT GAC TGG-3'; ablX2 sense upstream 5'-CCT TCA GCG GCC AGT AGC AT-3'; CML bcr upstream 5'-ACA GCA TTC CGC TGA CCA TC-3'; CML abl antisense detection 5'-TAT GCT TAG A(;T GTT ATC TCC ACT-3'.

PCR Method of Hogewisch

Method used for bcr-abl PCR by Hogewisch-Becker et al. [J. Biol. Chem. Suppl. 188, 289 (1989)] on cases CML-3a, CML-3b, and CML-6. The oligonucleotide primers used were as follows: sense primer (upstream of bcr) 5'-AGG GTG CAC AGC CGC AAC GGC-3'; antisense primer (abl)5'-CGC TTC ACT CAG ACC CTG AGG-5'; probe for the identification of bcr3/abl2 junction sequence 5'-GAA GGG CTT TTG AAC TCT G-3'; probe for the identification of bcr2abl2 junction sequence 5'-GAA GCG CTT CTT CCT TAT-3'. Exon 3 of bcr is joined to abl exon 2 if a 314-bp fragment is amplified. Exon 2 of bcr is joined to abl exon 2 if a 239-bp fragment is amplified.

Southern Blot

Southern blot analysis on case CML-4 showed a rearranged Bgl II band using an OSI Transprobe-1 Kit (Oncogene Science catalog no. TP88).

Construction of a PEM Library (Maniatis, 1982)

The PEM library was made from human placental DNA. This is considered to be "normal" DNA. The DNA was prepared by partial MbaI digestion, size selection, and ligation to the vector EMBL3 at the BAMHI site.

The vector inserts in the 15–20 kb size range. The insert can be excised with SalI. The Bam site is usually lost due to ligation to MbaI.

The insert-containing phage has no EcoRI sites, whereas the wild-type phage has EcoRI sites.

Phage may be grown on NM539 media. 10–12 plates of 50,000 clones each are generally screened.

The library has been amplified. This generally leads to a titer drop of about 10-fold.

Protocol for Construction of a Cosmid Library (Maniatis, 1982)

A cosmid library was plated on amphicillin plates and amplified on chloromphenical.

Phage were grown on filters in 20 plates. The titre was about 8x10 screened colonies per plate. Normally 2–4 of a few genome equivalents were obtained.

Replica plating was performed, 2 sets of each plates were screened with a probe.

To prepare a probe, generally an insert was cut out of a plasmid so that the plasmid sequences did not cross hybridize.

An ECQ fragment 228–1–2 (a 3' probe) was made by this method. It was then nick translated. An oligonucleotide may also be prepared synthetically. The duplicate filters were treated to fix and denature them by techniques known to those of skill in the art.

Each filter was treated on a column with

0.5M NaOH

1.5M NaCl

and moved to

1M TRIS ph 7.5

then into

0.5 TRIS 7.5

1.5M NaCl

Filters were blotted with 13M filter paper and dried at 68° C.

Nitrocellulase paper not nylon was preferred

A pre-hybridization mix was used to screen the library at 68° C.

The label was 7×10^6 CPM p^{32} for the probe.

Hybridization was performed at 42° C.

Washed in 2× SSC

5% SDS

(68%) for 2–3 hours

The filters were exposed until colonies were visible (~3 days)

Positive colonies were scored.

Colony re-purification was performed by rescreening in a similar procedure to the above.

Phage

2 clones were isolated from a phage library (human genome library of anonymous human placenta).

EMBO 1 partial digestion of DNA was performed.

DNA size was selected on a sucrose gradient to be in the 15–20 kb average size.

The fragments were ligated into the BAM HI site of the EMBO 3 vector (Stratogene or Promega).

The PEM12 clone was plated and the library screened 50,000 clones with 2 probes. The result was 2 clones.

These were screened with a BCR exon probe.

The insert was $\sim16^6$ kb.

Colony Biotechnology Systems, NEM Research Products Plaque Screen Cat NEF 978/978A 978X / 978Y

U.S. Pat. No. 4,455,370 du Pont de Nemours.

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SEQUENCE LISTING

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(1) GENERAL INFORMATION:

   (iii) NUMBER OF SEQUENCES: 4


(2) INFORMATION FOR SEQ ID NO:1:

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

    (ii) MOLECULE TYPE: DNA

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

AGGGTGCACA GCCGCAACGG C                                              21


(2) INFORMATION FOR SEQ ID NO:2:

     (i) SEQUENCE CHARACTERISTICS:
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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA

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

CGCTTCACTC AGACCCTGAG G                                              21


(2) INFORMATION FOR SEQ ID NO:3:

     (i) SEQUENCE CHARACTERISTICS:
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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear
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    (ii) MOLECULE TYPE: DNA

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

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(2) INFORMATION FOR SEQ ID NO:4:

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          (B) TYPE: nucleic acid
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What is claimed is:

1. A composition comprising at least two probes, each labeled with a distinguishable label, for detecting a chromosomal aberration involving the BCR and ABL genes, said chromosomal aberration having an ABL gene side and a BCR gene side, wherein one of said probes hybridizes to the ABL gene side of said chromosomal aberration and the other of said probes hybridizes to the BCR gene side of said chromosomal aberration, wherein said probes hybridize to an aberrant chromosome wherein said probes are of sufficient length to be specifically detected in cytogenetic analysis.

2. A composition comprising at least two probes for detecting a chromosomal aberration, each probe labeled with a distinguishable label, wherein one of said probes hybridizes to a part of the ABL gene on one side of said chromosomal aberration and the other of said probes hybridizes to a part of the BCR gene on the other side of said chromosomal aberration, wherein said probes hybridize to an aberrant chromosome wherein said probes are of sufficient length to be specifically detected in cytogenetic analysis.

3. The composition of claim 2 wherein said probes hybridize within approximately 800 kb of each other in said aberrant chromosome.

4. The composition of claim 1 wherein the labels comprise fluorescent labels.

5. The composition of claim 4 wherein the fluorescent labels are distinguishable under a microscope as different colors.

6. The composition of claim 1 wherein the probes hybridize with chromosomal DNA in situ in cells.

7. The composition of claim 6 wherein the cells comprise those in interphase of mitotic division.

8. The composition of claim 7 wherein the probes after hybridization are juxtaposed as doublets if a chromosomal aberration is present.

9. The composition of claim 1 wherein one of said probes is capable of hybridizing to at least a portion of the last exon of the ABL gene and the other of said probes is capable of hybridizing to at least a portion of exon I of the BCR gene.

10. The composition of claim 8 wherein the chromosomal aberration is further defined as comprising a translocation, said translocation formed by breakpoints which occur on the long arms of human chromosomes 9 and 22.

11. The composition of claim 10 wherein the translocation breakpoints are further defined as occurring at the locations designated t(9;22) (q11;q34).

12. The composition of claim 11 wherein the translocation breakpoints are further defined to occur in the BCR and ABL genes respectively, and a fusion gene is formed by the translocation, and said fusion gene comprises portions of the BCR and ABL genes.

13. The composition of claim 12 wherein the fusion gene encodes a protein p190.

14. The composition of claim 6 wherein the cells comprise a sample of human tissue.

15. The composition of claim 14 wherein the human tissue sample comprises peripheral blood.

16. The composition of claim 15 wherein the human tissue sample comprises bone marrow.

17. The composition of claim 6 wherein the cells comprise a sample of cultured cells.

18. The composition of claim 1 wherein one of said probes is capable of hybridizing to the major breakpoint cluster region (M-bcr) of chromosome 22.

19. The composition of claim 1 wherein one of said probes is capable of hybridizing to the first exon of the BCR gene.

20. The composition of claim 1 wherein one of said probes is capable of hybridizing at least a part of the last exon of the ABL gene.

21. The composition of claim 12 wherein the fusion gene encodes either of two proteins p190 and p210.

22. The composition of claim 21 wherein the presence of said fusion gene is diagnostic or prognostic for acute lymphocytic leukemia (ALL).

23. The composition of claim 21 wherein the presence of said fusion gene is diagnostic or prognostic for chronic myelogenous leukemia (CML).

24. A kit for the detection of chromosomal aberrations, comprising a first and second nucleic acid probe, each labeled with a distinguishable label, said first probe capable of specifically hybridizing to a part of the ABL gene on one side of said chromosomal aberration and said second probe capable of specifically hybridizing to a part of the BCR gene on the other side of said chromosomal aberration, wherein said probes hybridize to an aberrant chromosome wherein said probes are of sufficient length to be specifically detected in cytogenetic analysis.

25. The composition of claim 1 wherein the aberrant chromosome is the Philadelphia chromosome.

* * * * *

EXHIBIT 2

US006414133B1

(12) **United States Patent**
Dietz-Band et al.

(10) **Patent No.: US 6,414,133 B1**
(45) **Date of Patent: Jul. 2, 2002**

(54) **MULTIPLE FUSION PROBES**

(75) Inventors: **Jeanne Dietz-Band**, Keedysville; **Wang-Ting Hsieh**, Bethesda; **John F. Connaughton**, Laytonsville, all of MD (US)

(73) Assignee: **Ventana Medical Systems, Inc.**, Tucson, AZ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/170,630**

(22) Filed: **Oct. 13, 1998**

(51) **Int. Cl.**[7] **C07H 21/04**; C12Q 1/68; C12P 19/34

(52) **U.S. Cl.** **536/24.3**; 536/24.31; 536/24.32; 536/24.33; 536/23.1; 435/6; 435/91.1

(58) **Field of Search** 536/24.3, 25.3, 536/23.1, 24.31, 24.33, 24.32; 435/6, 91.1, 2, 91.2, 810

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Primary Examiner—W. Gary Jones
Assistant Examiner—Cynthia Wilder
(74) *Attorney, Agent, or Firm*—Huw R. Jones; John E. Tarcza; Ann S. Hobbs

(57) **ABSTRACT**

The invention is directed to a DNA probe set, the probe set comprising a first probe set and a second probe set, the first probe set being sufficient in length and substantially complementary to an entire breakpoint region of a first DNA and nucleotides breakpoint region but less than an entire chromosome such that the first probe set will hybridize to both sides of the breakpoint region regardless of whether the first DNA has been broken in the breakpoint region and either end fused to another DNA, and the second probe set being sufficient in length and substantially complementary to an entire breakpoint region of a second DNA and nucleotides on both sides of the breakpoint region but less than an entire chromosome such that the second probe set will hybridize to both sides of the breakpoint region regardless of whether the second DNA has been broken in the breakpoint region and either end fused to another DNA. Diagnostic kits utilizing the probe sets of the invention are also claimed.

19 Claims, 10 Drawing Sheets

(1 of 10 Drawing Sheet(s) Filed in Color)

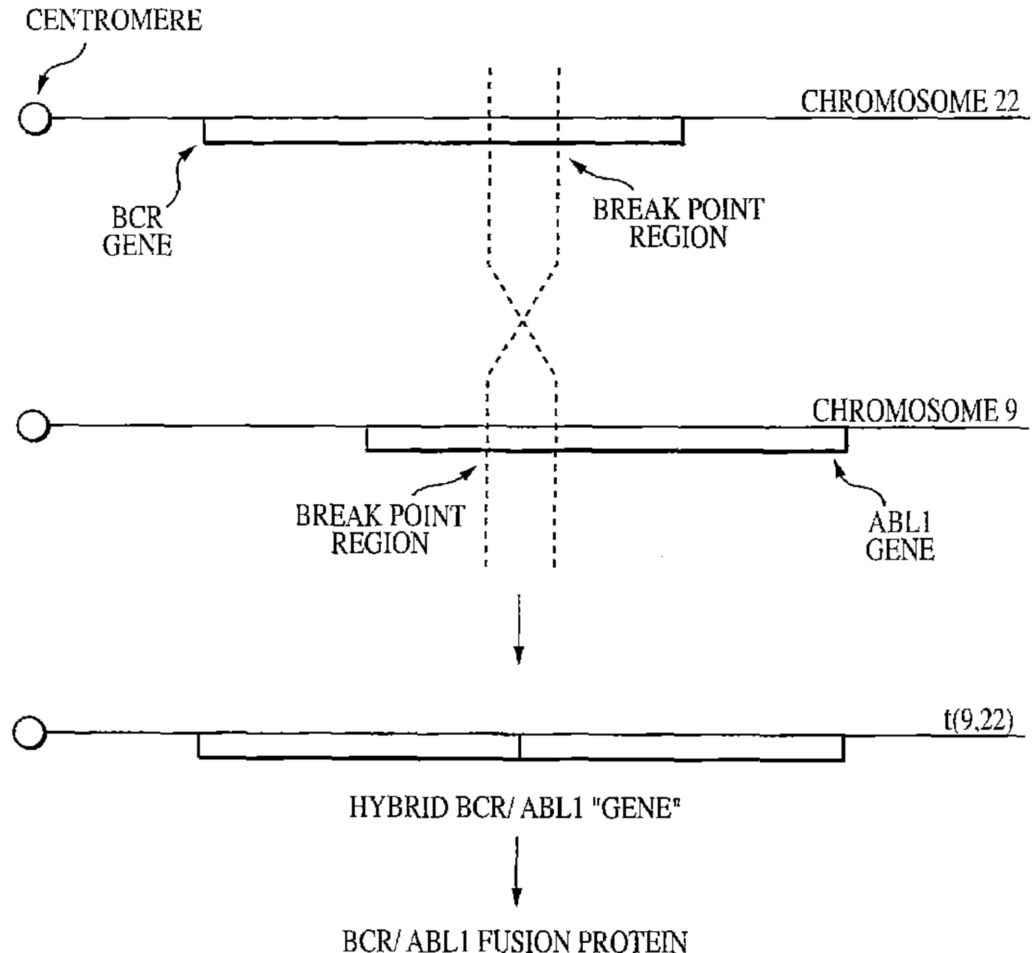
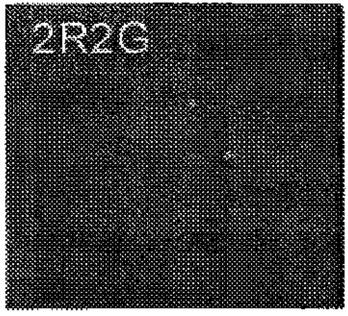
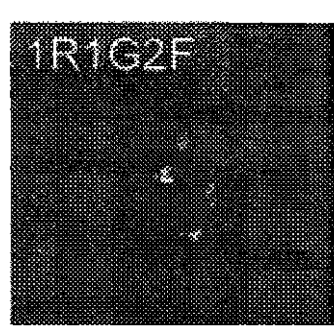


FIG. 1



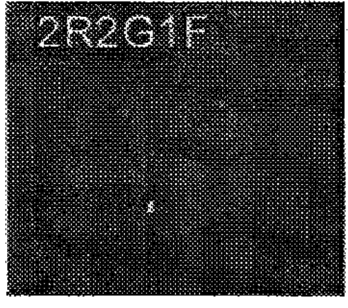
SCORING KEY:
R = RED SIGNAL = BCR
G = GREEN SIGNAL = ABL1
F = YELLOW SIGNAL BCR/ABL1 FUSION

FIG. 2a



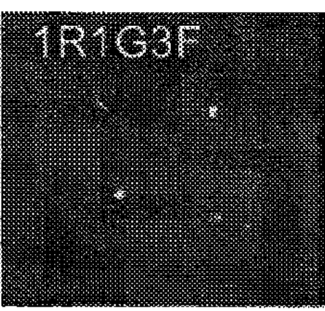
SCORING KEY:
R = RED SIGNAL = BCR
G = GREEN SIGNAL = ABL1
F = YELLOW SIGNAL BCR/ABL1 FUSION

FIG. 2b



SCORING KEY:
R = RED SIGNAL = BCR
G = GREEN SIGNAL = ABL1
F = YELLOW SIGNAL BCR/ABL1 FUSION

FIG. 2c



SCORING KEY:
R = RED SIGNAL = BCR
G = GREEN SIGNAL = ABL1
F = YELLOW SIGNAL BCR/ABL1 FUSION

FIG. 2d

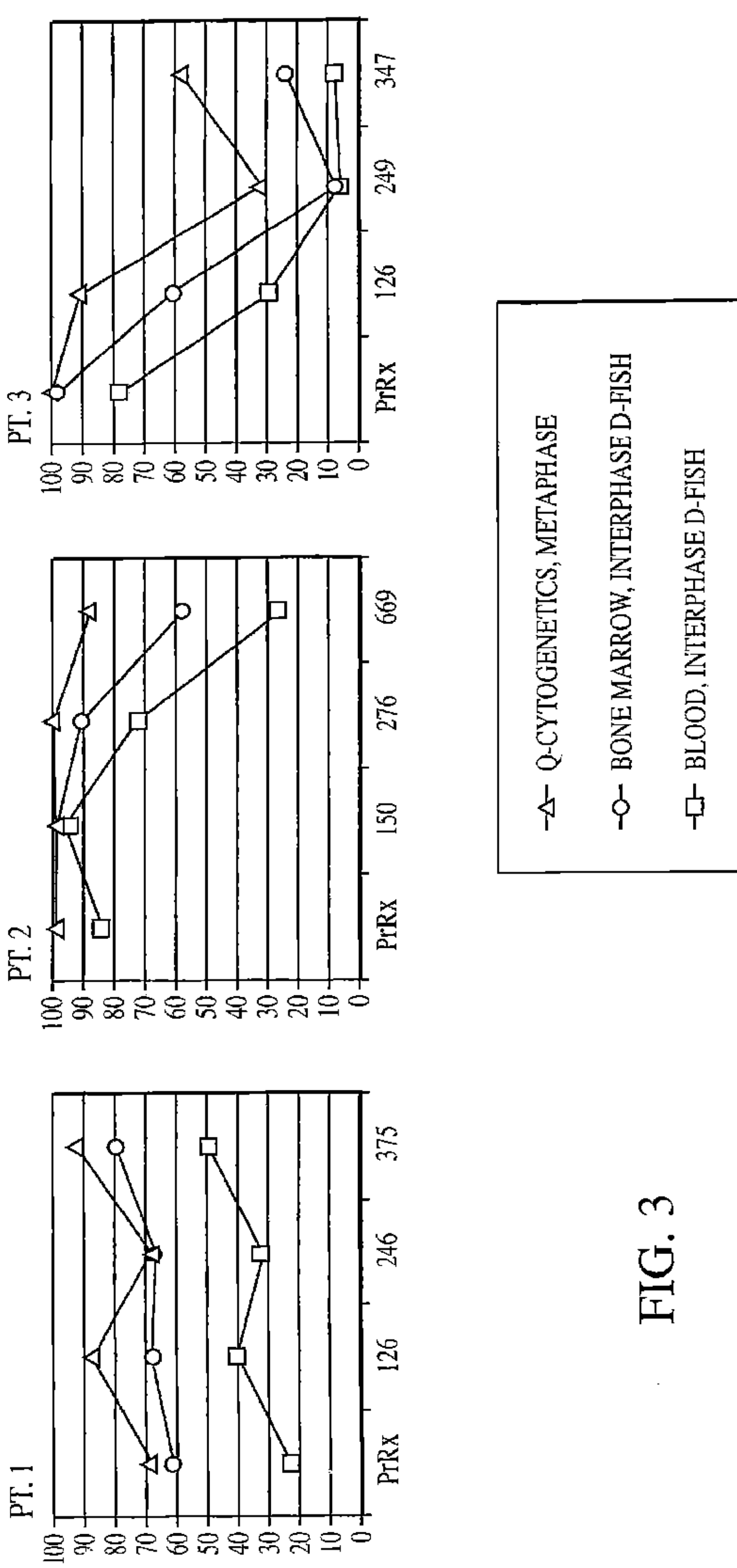


FIG. 3

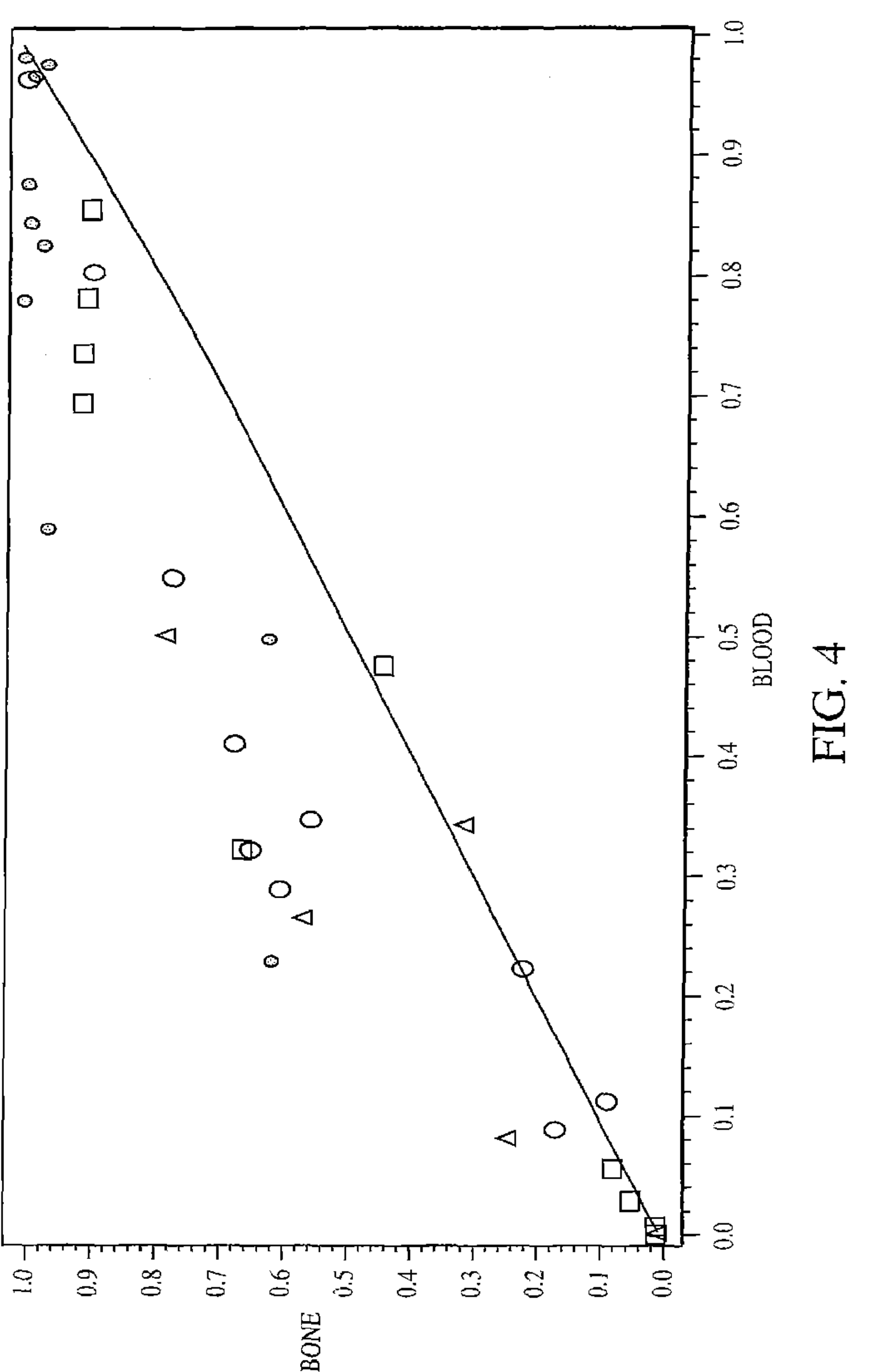


FIG. 4

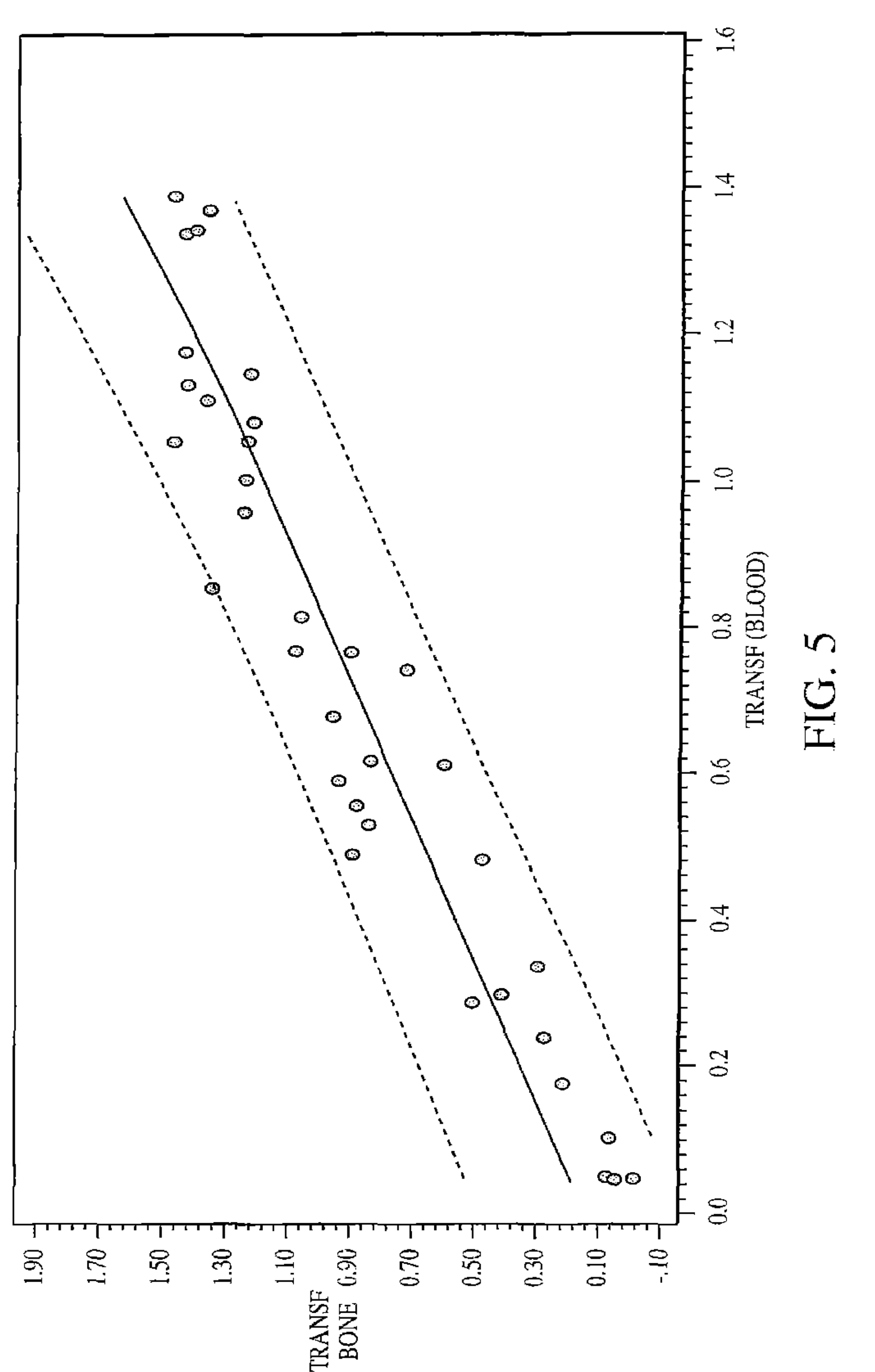


FIG. 5

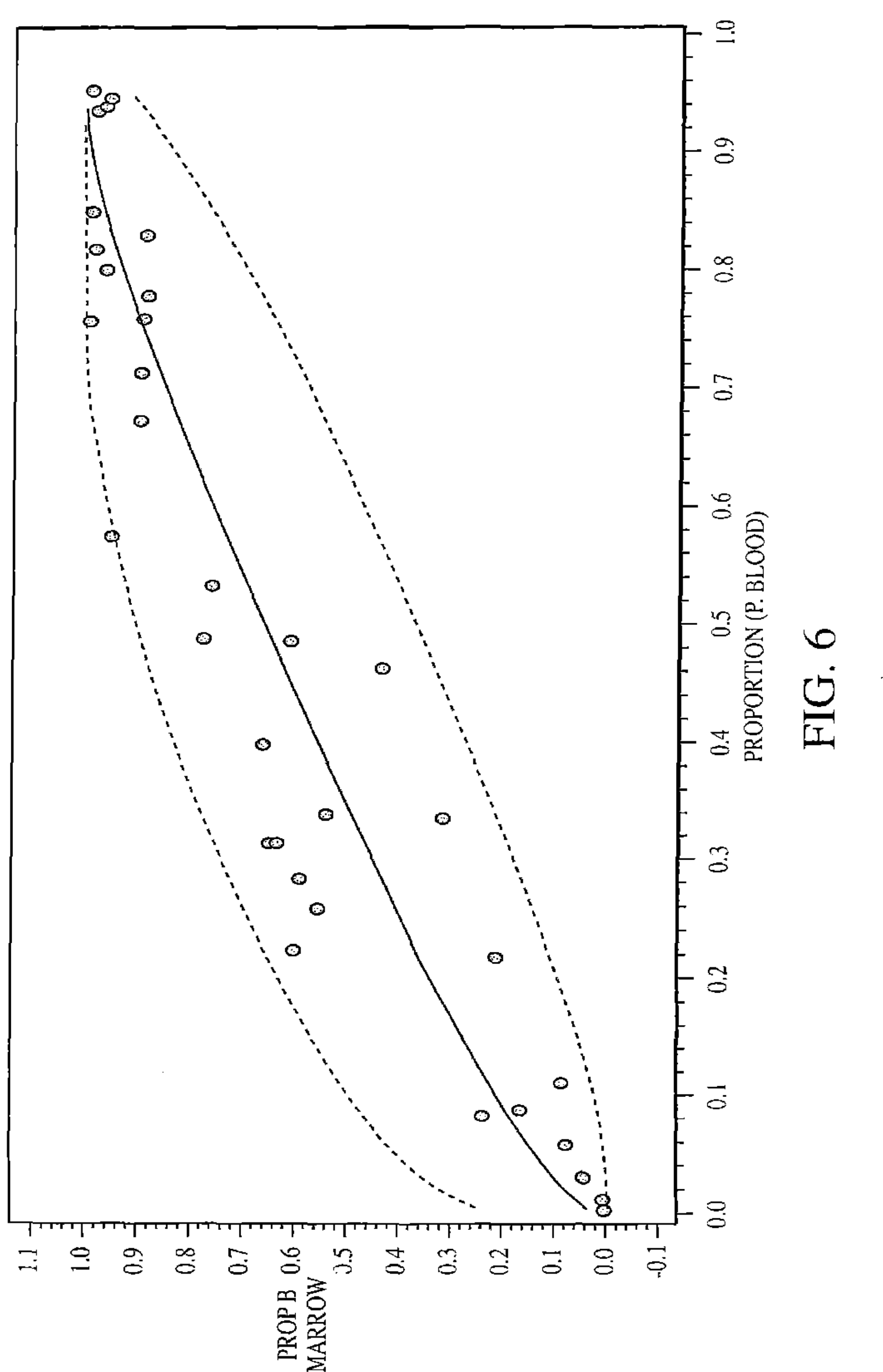


FIG. 6

TABLE 1: BCR SUMMARY

MAPPING OF BCR CLONES BCR REGION CONTAINS 152,141 BP PUBLISHED SEQUENCE		PRIMER PAIRS							
NAME OF CLONE	CLONE TYPE	BCR26/27	BCR13/14	BCRA/B	BCRC/D	BCRE/F	BCR11/12	BCR17/18	CLONE SIZE (Kb)
OCB 1004	PAC			+	+	+	+	+	214
OCB 1005	PAC				-	-	+	+	105
OCB 1001	PAC	+	+	-	-				154
OCB 1002	PAC	+	+	+	-				127
OCB 1003	PAC	+	-						147

+ : THE CLONE CONTAINS THE SEQUENCE AMPLIFIED BY THIS PRIMER SET.
- : THE CLONE DOES NOT CONTAIN THE SEQUENCE AMPLIFIED BY THIS PRIMER SET.

FIG. 7

TABLE 2: ABL SUMMARY

MAPPING OF ABL CLONES

3 SEQUENCES ARE AVAILABLE:

HSABLGR1, 35,692 BP, 5' ABL, EXON1B(29132-29267)/INTRON 1B(29268-35692)
HSABLGR2, 59,012 BP, PARTIAL INTRON 1B
HSABLGR3, 84,539 BP, INTRON 1B(1-37824)/EXON1A TO EXON10 AND POLYA

NAME OF CLONE	CLONE TYPE	PRIMER PAIRS								CLONE SIZE (Kb)
		ABL5/6	ABL3/4	ABLa/b	ABLe/f	ABL9/10	ABLc/d	ABL7/8	ABL19/20	
OC3002	P1	-	-	+	+	+	-			88
OCA1003	BAC	+	+	+	-					101
OCA1001	PAC	+	-	-		-				181
OCA1004	YAC				-	+	+	+	-	250
OCA1005	PAC						-	+	+	186
OCA1006	PAC						-	+	+	153
OCA1007	PAC						-	-	+	138

+ : THE CLONE CONTAINS THE SEQUENCE AMPLIFIED BY THIS PRIMER SET.
- : THE CLONE DOES NOT CONTAIN THE SEQUENCE AMPLIFIED BY THIS PRIMER SET.

FIG. 8

TABLE 3

		500 NUCLEI				6,000 NUCLEI				HYPERMETAPHASE BONE MARROW
		BONE MARROW		PERIPHERAL BLOOD		BONE MARROW		PERIPHERAL BLOOD		
PT	SPEC	%ABN	ABN NUCLEI	%ABN	ABN NUCLEI	%ABN	ABN NUCLEI	%ABN	ABN NUCLEI	% ABN (PH POSITIVE/ METAPHASES ANALYZED)
4	3	0.6%	3	0.2%	1	0.22%	13	0.10%	6	0.0%(0/27)
4	4	0.4%	2	0.2%	1	0.23%	14	0.08%	5	0.0%(015)
4	5	0.0%	0	0.0%	0	NA	NA	0.13%	8	0.0%(1/169)
5	3	0.6%	3	1.0%	5	1.30%	78	0.95%	57	0.0%(0/136)
5	4	0.0%	0	0.2%	1	0.05%	3	0.12%	7	0.0%(0/126)
NORMAL CUTOFF		>0.8%	>4	>0.8%	>4	>0.079%	>4	>0.079%	>4	

FIG. 9

TABLE 4

		MEAN PROPORTIONS (+SE) ORIGINAL SCALE		ADJUSTED MEAN DELTA (+SE)
SAMPLE	PTS	BONE MARROW	BLOOD	(TRANSFORMED SCALE)
DX	10	0.91 (±0.05)	0.75 (±0.08)	0.165 (±0.047)
4 MOS	10	0.56 (±0.10)	0.41 (±0.09)	0.177 (±0.042)
8 MOS	10	0.49 (±0.13)	0.39 (±0.11)	0.150 (±0.042)
12 MOS	6	0.32 (±0.13)	0.20 (±0.08)	0.181 (±0.054)

FIG. 10

MULTIPLE FUSION PROBES

FIELD OF THE INVENTION

The invention relates to improved polynucleotide probe configurations for detecting structural abnormalities that result from chromosome breakage and rearrangement, particularly as used in the detection of several types of genetic disorders related to cancer and other diseases. The invention further relates to an improved method of detecting translocations using probe sets which span each breakpoint region associated with a translocation and the regions on both sides beyond the 3' and 5' ends of each breakpoint region.

BACKGROUND OF THE INVENTION

A number of inherited genetic diseases and types of cancer have been linked to chromosomal translocation events which result in the fusion of two genes which do not occur together in the normal genome. Certain conditions involve translocations which frequently occur at the same or very near location. The chromosome regions where frequent breaks occur are called breakpoint regions.

One of the best known examples of a clinically important translocation is the Philadelphia Chromosome which results from a break in the ABL1 gene on distal chromosome 9q and the BCR gene on proximal chromosome 22q {t(9;22)} (FIG. 1). The breakpoints within the ABL1 gene may occur throughout a region spanning more than 175 kb upstream from exon II while the breaks in chromosome 22 are clustered into two areas of the BCR gene, termed the major breakpoint cluster region (m-bcr) and the minor breakpoint cluster region (M-bcr) (Kurzrock et al, *New England Journal of Medicine*, 319:990 (1988)). The Philadelphia Chromosome occurs in most cases of Chronic Myelogenous Leukemia (CML) and some cases of Acute Lymphocytic Leukemia (ALL). Other important translocations include, but are not limited to, t(8;21) in Acute Myelogenous Leukemia, t(8:14) in Burkett's Lymphoma and pre-B-cell Acute Lymphoblastic Leukemia, t(1:14), t(7:9), t(7:19), t(11:14), t(10:14) and t(7:9) in T-acute Lymphoblastic Leukemia, t(15;17) in Acute Myelogenous Leukemia (AML) and t(15:17) Acute Promyelocytic Leukemia (PML). Solid tumors include, t(9;22) in Ewing's Sarcoma, t(15:16), and hereditary diseases associated with translocations include a number of mental retardation associated syndromes. It is likely that other conditions are caused by subcriptic translocations or other structural aberrations which are yet to be determined and are too small to be noticed by standard cytogenetics.

Multiple genetic testing methods have been developed for use in diagnosis, monitoring of minimal residual disease and/or response to therapy during clinical practice. However, no single technique has been developed that can accurately detect and quantify disease at diagnosis and throughout treatment. Conventional quantitative cytogenetics and G-banding analysis is cumbersome and can only be applied to cycling cells (Lion, Leukemia 10: 896 (1996)). In practice, the sensitivity of conventional cytogenetics is dependent upon the number of good metaphase cells which can be evaluated. In the example of cancers caused by neoplastic cells in the bone marrow, obtaining large numbers of good metaphase cells from bone marrows of patients is difficult.

More recently, the assay technique in situ hybridization (ISH), particularly fluorescent in situ hybridization (FISH) (Pinkel et al, *Proc. Natl. Acad. Sci., U.S.A.* 83:2934–2938 (1986)) has been of assistance in detecting translocations. FISH allows the analysis of individual metaphase or interphase cells, thereby eliminating the need to obtain and assay cycling cells. It is therefore possible to use nondividing tissue, including bone marrow and peripheral blood cells in a diagnostic or prognostic analysis.

In the field of detecting the Philadelphia Chromosome, a commonly used method for detection of ABL1/BCR fusion utilizes differently labeled probes for BCR and ABL1, and detects a single ABL1/BCR fusion (or closely linked) signal in cells with a Ph chromosome. (This method is referred to for convenience as S-FISH.) An example of this technique is Tkachuk et al, *Science* 250: p. 559–562 (1990) where one fluorescently labeled probe hybridized to part of the ABL1 gene and a second fluorescently labeled probe hybridized to part of the BCR gene.

The probes in commercial single FISH test kits do not span the entire length of each translocation breakpoint but rather are designed to bind to one portion of each gene, i.e. sometimes overlapping or adjacent to a breakpoint region, sometimes many kilobases away and sometimes both (See FIG. 1 of Tkachuk et al for example). Normal chromosomes 9 and 22 each bind one probe, which is specific to that chromosome. The Philadelphia Chromosome, both probes hybridize at the fusion site bringing both labels in close proximity so as to usually form a color shift or fusion near proximity/signal. Because the exact breakpoint may vary, the two probe labels may not come sufficiently close to form a fusion label. Likewise for probes useable to detect the t(8;21) translocation in Acute Myelogenous Leukemia (AML).

Using the probe configuration above, the following detection method for the Philadelphia Chromosome using FISH has been used: the ABL1 gene probe is labeled using a probe containing one hapten or fluorophore (for example, FITC) and the BCR gene probe is labeled using a probe containing another hapten or fluorophore (for example Rhodamine). After hybridization and detection, a normal chromosome 9 shows the green signal and a normal chromosome 22 shows a red signal. A normal cell would therefore exhibit two red signals and two green signals. A cell containing a Philadelphia chromosome has one red and one green signal for the unaffected homologues of chromosomes 9 and 22 and one white, yellow or closely linked pair of signals that results from the close proximity of the labeled probes hybridized to the translocated BCR and ABL1 genes, the so-called fusion signal.

However, the probes used heretofore in this method have not been constructed so as to specifically bind and detect the second fusion site for the reciprocal translocation event. Thus, the S-FISH method detects only one of the abnormal chromosomes resulting from the translocation event, the Philadelphia chromosome.

In another method using labelled probes to detect ALL gene rearrangements in solid tumors, a probe set was designed so that the two probes lie adjacent to each other on the normal chromosome, but split apart and move to the two different abnormal chromosomes if the translocation has occurred (Croce, U.S. Pat. No. 5,567,586, hereby incorporated by reference). In this method the probes are designed to be complementary to sequences in the translocation region on one chromosome. In this method, the fluorescent probes produce a single spot on the normal chromosome, but appear as two distinct spots when translocation has occurred.

The same format has been used for other assays for detecting other translocations such as t(8:21) in Acute

Myeloid Leukemia (AML). For example, Le Beau, Blood 81: 1979–1983 (1993), and Sacchi et al, Cancer Genetics and Cytogenetics 79: 97–103 (1995) and Fischer et al, Blood 88: 3962–3971 (1996).

SUMMARY OF THE INVENTION

It is an object of the invention to provide methods with increased sensitivity and accuracy for detecting chromosome translocations and other structural rearrangements which result in more than one abnormal fusion site in the genome.

It is a further object of the invention to provide probes and probe sets which are useful in detecting reciprocal genetic translocations according to the methods of the invention.

It is another object of the present invention to detect cancer, inherited disease, susceptibility to inherited disease or a carrier of a fused gene for an inherited disease wherein the condition results from a chromosomal translocation in one or more cells. This is particularly beneficial when the diagnosis, prognosis, monitoring for residual disease and response to therapy in cancer or other disease is dependant upon the quantity of abnormal cells as an indicia of the disease state and/or response to treatment.

It is also an object of the invention to provide a means of constructing such probes and probe sets, which will detect reciprocal fusions resulting from chromosomal translocations and will accordingly be useful in diagnosis, prognosis, monitoring of residual disease and response to therapy when reciprocal chromosome translocations are present.

It is still another object of the present invention to provide diagnostic test kits which can be used by any cytogenetist or other trained individual to detect multiple fusion events which result from structural rearrangement of the genome.

Probes and probe sets of the present invention have the characteristic of encompassing the entire breakpoint region and a region on each side of the breakpoint region on each chromosome for the reciprocal translocation event of interest and are capable of detecting such translocations with much greater sensitivity than the probes and probe sets which were previously known.

A particularly preferred probe set and method is used for detecting the Philadelphia chromosome and its corresponding derivative chromosome as companion indicators of CML and some other cancers such as ALL. One functional probe is designated P5161-DC, described hereinbelow. Another example is for detecting the AML1/ETO gene fusion in AML.

The use of specifically designed probe sets by the method of the present invention has allowed the clinician to assess physical information regarding all fusion events associated with a defined structural rearrangement in a cell. For example, using the standard detection method of fluorescence in situ hybridization (FISH) it has been demonstrated that these probe sets provide the following advantages over traditional testing methodologies for detecting the same translocation.

1. Unlike traditional single fusion probe sets, probe sets which detect multiple, derivatives of a structural rearrangement have the ability to detect much lower copy numbers of abnormal cells thereby providing greater improved diagnostics using FISH assays
2. The ability of the probe sets to derive necessary information from cells in interphase, Thereby rivaling the sensitivity of metaphase cells in conventional cytogenetics.
3. Specifically, increased sensitivity has been demonstrated with multiple fusion probes used in interphase FISH analysis which is at least as sensitive as Q-cytogenetics (the previous gold standard) for monitoring bone marrow or peripheral blood cell populations for minimal residual disease and response to therapy.
4. Greater sensitivity allows the use of peripheral blood instead of invasive and painful bone marrow samples from patients for routine testing, to monitor for minimal residual disease and response to therapy.
5. By detecting high and low copy numbers of gene fusions, the present invention can be used for diagnosis and monitoring throughout the course of the disease thereby avoiding traditional multiple assay-type testing methodologies.
6. Simplified sample requirements and testing provides further benefits in cost and patient well being.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

FIG. 1 shows a schematic drawing of a BCR/ABL1 translocation and probes constructed according to the invention.

FIG. 2A shows the appearance of a normal cell after testing with probe P5161-DC using the methods of the invention. The two red signals designate the BCR genes on chromosome 22 and the two green signals designate the ABL1 genes on chromosome 9.

FIG. 2B shows the appearance of a cell containing a BCR/ABL1 translocation after testing with probe P5161-DC using the methods of the invention. One red, one green and two fused signals denoting both of the reciprocal translocation events are present.

FIG. 2C also shows the appearance of a cell containing a BCR/ABL1 translocation after testing with probe P5161-DC using the methods of the invention. Two red, two green and one fused signals are present in this example. While two fusion signals are usually detected, because of the physical configuration of the gene and the relaxation of the heterochromatin in interphase, a red and a green signal may appear to be closely linked but not quite overlapping. Note the two signals at the lower end of the field which are not quite fused. This configuration is believed to represent the fused portion of a translocation event.

FIG. 2D shows the appearance of a cell containing a BCR/ABL1 translocation after testing with probe P5161-DC using the methods of the invention. One red, one green and three fused signals are present. This cell contains an additional Philadelphia chromosome.

FIG. 3. Percentage of Ph positive cells (Y-axis) prior to therapy and during treatment at approximately 4 month sampling intervals (X-axis in days) in bone marrow by Q-cytogenetics and D-FISH, and blood by D-FISH.

FIG. 4. Relationship between the percentage of Ph positive cells for paired-sets of bone marrow (Y-axis) and peripheral blood (X-axis).

FIG. 5. Linear regression analysis of the (transformed) proportion of abnormal cells from bone marrow on the (transformed) proportion from peripheral blood from FIG. 4. Dashed lines are the 95% prediction interval.

FIG. 6. Results of linear regression analysis but transformed to original scale of proportions of abnormal cells for bone narrow (Y-axis) versus peripheral blood (X-axis). Dashed lines represent the 95 prediction internal for a bone narrow prediction given a specific peripheral blood score.

FIG. 7. BCR map and summary of probe listed as Table 1.

FIG. 8. ABL1 map and summary of probe listed as Table 2.

FIG. 9. Data and comparison of different techniques for assaying for the Philadelphia chromosome, listed as Table 3.

FIG. 10. Data comparing bone marrow and blood samples for monitoring the disease state and response to therapy, listed as Table 4.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is an assay and reagents therefore which may be packaged into a simple test kit. The reagent comprises two probes, the first complementary to and encompassing the entire breakpoint region on a first chromosome as well as both upstream and downstream regions from the breakpoint region. As such, the first probe will hybridize to a normal first chromosome as well as both fragments of the first chromosome which have undergone a translocation or break and may be rearranged elsewhere. The second probe is the same as the first except for spanning the entire breakpoint region, as well as both upstream and downstream regions on the second chromosome. Kits may also be constructed for multiple translocation events or having more than two chromosomes involved according to the methods of the present invention.

Each probe is detectable when hybridized to the target DNA, preferably by being labeled with a unique detectable label that can be either directly or indirectly detected. The labeling may be by covalent bonding or other affinity attachment. Each polynucleotide comprising a probe is labeled with the same label and each probe has a detectably different label from other probes in the mixture. As such, one can easily detect each normal chromosome potentially involved in the translocation as well as fusions between the two or more breakpoint regions which are detected as a fusion signal.

The different construction of multiple fusion probes provides numerous advantages over two similarly labeled single fusion probes hybridizing to different locations. Some of these advantages were not apparent until after testing the probes on biological samples. Thus, by constructing probes complementary to the entire breakpoint regions, as in D-FISH, one achieves certain advantages over conventional S-FISH.

In the present invention's improved method, designated D-FISH, fusion signals can be detected in each cell as an indicator of the presence of a reciprocal translocation. The sensitivity of the method using the available S-FISH probes has not been sufficient in the past to detect very low levels of translocations which are found in the peripheral blood cells or bone marrows of many patients. Specifically, commercially available S-FISH detects about 70–75% of patients actually positive for the Philadelphia chromosome. Actual positive results were necessarily determined by sequencing, PCR amplification or Southern blotting. By contrast, using D-FISH with the probes of the present invention detects greater than 99% of patients actually positive. This data rivals or exceeds standard Q-cytogenetics (See Dewald et al data). The improved detection indicates that the present invention should be accepted as the "gold standard" by which all other assays are compared.

This improved sensitivity is accomplished by using probes which are specifically developed to cover the entire breakpoint region of each chromosome involved in the translocation. In such a translocation, the labeled probe for a first chromosome is immediately adjacent to the labeled probe for a second chromosome thereby producing multiple fusion signals. By contrast, S-FISH employs probes which are designed to be complementary to one side of each of two breakpoint regions and therefore relies upon the detection of a single fusion event.

The new method presented here involves novel DNA probe constructs which are designed to target the length of each breakpoint region in a translocation event and additional DNA sequence beyond both the 3' and the 5' ends of each breakpoint region. When the breakpoint region occurs in a gene, it is preferable for the probes to encompass the entire gene and additional DNA sequence beyond both ends also. When used together, all reciprocal translocation events involving the target gene regions can be detected simultaneously in an interphase or metaphase cell. The probes are further designed to give easily visualized balanced signals in interphase cells.

The effectiveness of dual or multi-fusion probes is perhaps best demonstrated when the DNA probes are fluorescently labeled in different colors and hybridized to cellular DNA using the standard assay technique of fluorescence in situ hybridization (FISH) (Pinkel et al, *Proc. Natl. Acad. Sci., U.S.A.* 83:2934–2938 (1986)). Various types of DNA probe configurations have been used with FISH technology in an attempt to find a reliable and sensitive assay for interphase cell analysis. However all of these techniques provide either too many false positives, false negatives or simply lack the sensitivity to determine the presence of the translocation in interphase detection of minimal residual disease and/or response to therapy. Additionally, other techniques are cumbersome or expensive to test or require large quantities of hard-to-obtain biological material. By comparison, the Examples below show obtaining a result from a simple blood sample using conventional cytogenetic equipment with a high sensitivity and low error rate.

Balanced and non-reciprocal translocations may also be detected using the probe strategy and method of the present invention. Even inversions within the same chromosome may be detected as double fusions with the probe sets developed for the two breakpoint regions on the same chromosome. In such situations, one still has two breakpoint regions forming at least one fusion site for detection of a fusion signal. The same general principles apply either way. In accordance with the present invention, probes constructed in accordance with the general instructions provided herein are used to produce reagents and a method for detecting multiple breaks as well as any resulting fusions thereby determining the presence of such multiple breakage events simultaneously. The present invention may also be used for screening for chromosome breakage at multiple genome sites due to environmental factors, chemicals, radiation (diagnostic X-rays or radiation therapy or radiation exposure), biological agents etc.

The source of cells may be highly variable. If a cancer is being diagnosed or monitored, cells from the tumor site or removed from the tumor site may be used. For inherited diseases, readily available cells from tissues, blood, urine, feces, buccal scrapings, cervical and vaginal scrapings (PAP smear), body fluids, etc. may be used. For prenatal testing, fetal, amniotic, placental, cord, chorionic villus, and "cells" including sperm or egg for the situations of gamete "donation" or in vitro fertilization may be used.

The cells being tested may be in any phase, but metaphase and interphase are preferred.

While this application generally refers to humans and human diseases, persons of skill will appreciate the invention is useful in other settings. The present invention is equally applicable to other animals for agricultural or veterinary purposes as useful in the diagnosis, prognosis and monitoring of disease. If so desired, the present invention is applicable to determining translocations in plants as well. The present invention is also applicable to non-disease conditions where determining the presence of a translocation is important for plant and animal breeding such as to follow the presence of a trait throughout generations.

One standard method for plant breeding involves infecting the plant with Agrobacterium tumefaciens carrying a Ti plasmid which will integrate T-DNA into the plant chromosome. Transfection of the plasmid per se may also be used. A desired gene is typically incorporated into the T-DNA region, especially in a hormone gene if not already deleted. In such a situation, the plant chromosome has a breakpoint region for inserted T-DNA. Probes encompassing the plant chromosome breakpoint region and the two ends of the T-DNA or the desired gene may be used to determine whether plant cells contain the desired gene. The present invention results in considerable time savings compared to tissue culture and cultivation of the plant to maturity and testing for a trait caused by the desired gene.

Along the same procedural lines, the present invention may be used to assay for insertion of any other DNA into a specific chromosome site such as is desirable in gene therapy. During certain forms of gene therapy, added DNA is incorporated into the host chromosomes at specific locations. The present invention permits monitoring and provides proof of integration.

Viruses which integrate are biologically significantly different when their DNA is integrated into a host chromosome. Such an integration involves a breakage of the chromosome and a fusion of the viral DNA into the chromosome. Monitoring the integration is an important step in assaying for antiviral therapeutics, determining prognosis, etc. If the integration site or region is known, probes to that site and to the virus (or viruses if two are coinfecting) may be prepared and used according to the present invention.

False positives can occur in normal cells, for example when the BCR locus of chromosome 22 coincidently occurs very close to, behind or in front of the ABL1 locus of chromosome 9. Since the cell being viewed is three dimensional and usually in interphase, the chromosomes are freely moving within the cell nucleus permitting a random juxtaposition of signal. About 4% of normal slides have the two loci sufficiently superimposed on each other to cause the signals to appear fused using S-FISH.

However, when using D-FISH, two fusion signals typically occur as well as two normal signals. The percentage of normal cells with both ABL1 and both BCR loci coincidently superimposed is very small. Thus, the false positive rate in D-FISH is lower. Still further, normal cells displaying two fusion signals by chance would not display two normal signals as well, providing a further check against false positives.

As for reducing the false negative rate and increasing sensitivity, one potentially has twice as many fusion signals per cell which makes it easier to detect an abnormal cell.

The advantages of the present invention depends upon a number of factors, including the unique probe configuration, the number or percentage of affected cells, which may vary with individuals and disease states. For example, the methods described above typically require that about 1% of the cells be affected for accurate signal detection. This compares to a S-FISH assay requiring about 30% of the cells positive. If fewer cells are affected, an abnormal condition may not be detected.

While the Examples use microscopic identification of normal and abnormal chromosomes, other techniques may be used. For example, cells may be observed and determined to contain or not contain a translocation during flow cytometry or extracts may be taken and conventional DNA hybridization assays performed.

Several factors determine how large a probe construct should be. In the example of the BCR gene, for example, the probe would be sufficiently long to include both the major breakpoint cluster region and the minor breakpoint cluster region, as well as sequences beyond the gene. For other genes exemplified below, the breakpoint region is widely variable in size and requires probes of sufficient size unique to each application and may be determined by routine optimization. Generally, the probes will have a considerable length complementary to the adjacent non-breakpoint region for a normal or translocated fusion configuration. The length will depend on the particular translocation being detected. The length of each probe will further be manipulated to make visually balanced signals and/or enough to routinely cause a color shift when the signal is fused to a different label's signal. The length must accommodate all breaks, regardless of where in the breakpoint region the actual breakage and fusion occurs. Preferably, the length is also be sufficient to provide fusion signals of similar size throughout the target clinical population of cells, thereby increasing reliability and ease of interpretation.

Generally, the length of the probe sets will correlate to the length of the largest breakpoint region involved in a translocation event. Thereby, the balance of fusion signals in interphase cells is assured. The length may also be affected by the amount of label which can be incorporated on the probe. Considerable variance is acceptable, if there is optimization of labeling conditions for each probe being developed.

In one preferred embodiment of the invention, one probe of a probe pair is designed to be complementary to the ABL1 sequence (600 Kb) and the other probe is designed to be complementary to the BCR sequence (500 Kb). Individual cloned human DNA probes of varying lengths complementary to the ABL1 and BCR breakpoint regions were used collectively to make probes of appropriate length. A single clone may be used; however, if the insert is of sufficient size. In practice, probes are developed from overlapping "probe sets", consisting of several cloned DNA sequences which hybridize to and span the breakpoints on the relevant chromosomes.

Alternative techniques may be used other than FISH for probes of the present invention. For example, during the use of conventional blot assays, Southern and Northern, probes of the present invention may be optimized to be used in lieu of other labeling techniques. The probes of the present invention may also be used in developing assays in aqueous solution.

The probes of the invention may be detected after it is hybridized to the target DNA or RNA. This may be done by any technique which detects a probe containing double stranded DNA within the biological sample. If the remainder of the sample lacks significant double stranded regions, one may use chemicals which specifically bind to double stranded but not single stranded DNA or DNA/RNA. Examples include a labeled antibody to double stranded

DNA or RNA/DNA followed by detecting the label, ethidium bromide, SYBR green, an acridine dye (e.g. acridine orange), a protein or enzyme, etc.

The more preferred option is to have the probes labeled in order to provide a means of detection. Suitable labels include, but are not limited to, haptens and fluorophores, such as, FITC, Rhodamine and Texas Red as well as radioactive, chemiluminescent, bioluminescent, a metal chelator, quencher, enzyme, chemical modifications rendering the DNA detectable immunochemically or by affinity reactions, and other known labels. Many such suitable detection labels are known to persons of skill in the art of binding assays such as nucleic acid hybridization assays and immunoassays. When the label is a hapten, a receptor labeled directly or indirectly with an easily detectable substance, is employed before, with or after hybridization of the hapten labeled probe. When the label is a quencher, the absence of or reduced signal indicates the presence of the quencher.

Common ways to incorporate the label into the probe include nick translation, random priming or PCR amplification using a derivitized dNTP or NTP. Also post probe synthesis labeling and end labeling may be performed. The amount of label varies from one probe to another and the various uses for the probes. Too much labeling may actually cause a quenching effect. Typically about 1–25% of a nucleotide (A, G, C, or T) will be modified to incorporate a label into a DNA probe.

One of ordinary skill can choose appropriate labeling techniques, other colors or detection strategies which may vary depending on the particular translocation or other fusions being detected.

DEFINITIONS

As used herein, the term "probe" is intended to mean one or more polynucleic acids which hybridize specifically to a particular region of chromosome which is of interest. Depending on the size of the region, multiple polynucleotide molecules may be combined to comprise the probe. The number of polynucleotides will also be determined by whether the polynucleotides are synthesized chemically, by PCR, by plasmid, by cosmid, by yeast artificial chromosome (YAC) etc. Individual molecules comprising the probe may hybridize to overlapping portions of the chromosome of interest or may hybridize to physically linked regions separated from each other. These gaps may be sizable but should not be so large that upon hybridizing to a translocation locus in a cell, the probes are so far apart that they appear as non-associated signals and no fusion event can be reliably detected. For example, a 100 base pair gap is probably insignificant whereas a 1 Mb gap is too much to be acceptable. Note that the break may occur anywhere in the breakpoint region and therefore construction of the polynucleotide molecules composing the probe should be designed to accommodate breaks at the worst possible locations.

A probe need not have exact complementarity to the desired target, but should have sufficient complementarity to bind to the region of interest using the methods of the invention. To achieve this generally requires a matching sequence with at least 80%, preferably 95%, and most preferably about 100% complementarity to the target. Occasional polymorphisms may preclude true 100% complementarity in some individuals, particularly when the breakpoint does not occur in a coding sequence.

Accordingly, as used to refer to probes herein, the term "complementary" includes "substantially complementary" which is intended to refer to a probe which will specifically bind to the region of interest on a chromosome under the test conditions which are employed, and thus be useful for detecting and localizing the region. Complementarity will be extensive enough so that the probes will form specific and stable hybrids with the target DNA under the hybridization conditions used. Persons of skill in the art will be able to determine suitable sequences through the general knowledge available in the art, and by routine experimentation, using the examples set forth hereinbelow as guidelines.

A "cell" as used herein includes biological samples which were derived from cells. "Biological sample" includes all nucleic acid containing compositions where the nucleic acid (RNA or DNA, chromosome, viral, vector, mitochondrial . . .) was obtained from an individual organism or amplified from a nucleic acid obtained from an individual organism. The slide preparation procedure used in the Examples actually kills the cell and removes some of its components. However, the DNA remains. The term "cell" as used herein includes cellular components, extracts and other partial cellular components provided that they contain the nucleic acids. It is preferred that a reasonably complete set of the chromosomes remains or at least the DNA of the breakpoint regions and adjacent regions remains such that one can determine normal untranslocated DNA sequences from fused DNA sequences resulting from a translocation.

A "translocation" is the exchange of genetic material between two or more non-homologous chromosomes. This is frequently a reciprocal event where two chromosomes are simultaneously broken and the fragments are exchanged between the two chromosomes. Two new chromosome derivatives are created.

A piece of a chromosome may be broken twice and reincorporated in the same region in reversed order. This is called a inversion and is a subset of structural abnormalities caused by chromosomal breakage and rearrangement.

The present invention has many uses other than detecting reciprocal translocations such as detecting other chromosomal abnormalities caused by chromosomal breakage and rearrangement such as insertions, inversions, derivative chromosomes and possibly duplications and ring formations.

As used herein, the phrase "the entire breakpoint region" is intended to refer to a sequence or probe of sufficient length to include the entire region in which a particular break may occur. This region will vary with the particular structural aberration one wishes to detect. In rare instances where the boundaries of the breakpoint region may not be completely known or unclear, the breakpoint region is the region encompassing the distribution of two standard deviations of known breakpoints.

A "contig" is a collection of two or more overlapping cloned DNA fragments that when used together will extend the target region beyond that of using a singular cloned fragment. A contig refers to "contiguous" DNA fragments.

EXAMPLE 1: CONSTRUCTION OF BCR/ABL1 DUAL FUSION PROBES

The BCR/ABL1 dual fusion probes were assembled by screening through several different human libraries cloned into PAC, P1, BAC, and YAC vectors available from commercial sources, e.g. a CEPH library. The procedure included several rounds of sequencing and walking. These methods are known to persons of skill in the art and are described in various molecular procedure manuals such as PCR Protocols, A Guide to Methods and Applications, Innis et al, Academic Press, Inc. (1990) incorporated herein by reference.

Each round of screening included the following steps:

1. Synthesizing new PCR primers based on sequence information.
2. Establishing PCR conditions for the new primers.
3. Screening the libraries by either PCR (using primers) or DNA hybridization (by amplified fragments).
4. Selecting the positive clones.
5. Evaluating the positive clones by FISH. Verifying that the positive clone hybridizes to the correct region and does not show any cross hybridization.
6. Obtaining the end sequences of the insert of new clones by either direct sequencing or by sequencing the purified end fragment amplified by using a combination of Alu or other primers and vector end primers.
7. Comparing the new sequence to the existing sequence to establish the relative location of the new clone. New primers were then made from the new sequence outside the existing sequence.
8. Repeating steps 2–7 until the probes reached the appropriate length to include the entire breakpoint region and achieve the desired FISH signal intensity.
9. Establishing the relative locations of all clones in the final contig by STS mapping and estimating the size of the contig.

To obtain multiple fusion probes according to the invention, it is preferred that the probes cover both sides of the breakpoint and show not only good but also balanced signals in affected cells. For both BCR and ABL1 probes, screening was done for clones which collectively hybridize to the entire breakpoint region and both sides of the breakpoint region containing normal chromosomal DNA.

BCR:

The BCR dual fusion probe set is composed of 5 human PAC clones which are shown in Table 1, FIG. 7.

The BCR region contains a 152141 bp sequence published by GenBank. Three primer pairs were initially made, BCR a/b, BCR c/d, and BCR e/f, which correspond to the gene sequence at the −15 kb, −123 kb and −152 kb, 5' to 3' positions respectively. These primer sets were used to screen a P1 library by PCR and the amplified fragments were isolated and pooled to screen a PAC library by hybridization. Several positive P1 and PAC clones containing BCR gene sequences were obtained.

P1 Clone OC2001 was scored positive using primers BCR a/b. The end sequences of the insert were obtained. This clone has one end of the insert located in the BCR known sequence and one end outside the 5' end of known sequence. Primer set BCR 13/14 was synthesized using the new sequence information. Both PAC OCB1001 and OCB1002 were obtained by screening using BCR 13/14. The next round of screening was done by first sequencing the end sequences of the insert in PAC OCB1001, establishing the 5' and 3' positions of the ends and primers BCR 26/27 were made. PAC OCB1003 was acquired by screening the PAC library using the new primers BCR 26/27. This PAC is on the most 5' end of the contig.

PAC OCB1004 was obtained from the hybridization of PAC library using the pooled amplified DNA fragments generated by the BCR a-f primers described above. This clone covers almost all the BCR known sequence and also extends in the 3' direction.

From the PCR screening of the P1 library using primers BCR e/f, P1 clone OC2002 was obtained on the 3' end of the gene. Both ends of the insert were sequenced. This clone contains the BCR gene sequences from the 3' position, 109 kb into the gene, and extends further in the 3' direction from the end of the BCR gene. A new primer pair BCR 13/14 was made using the new 3' end sequence. PAC OCB1005 was obtained from the new screening which became the furthest 3' clone in the contig.

The size of the inserts of these individual clones are estimated by adding up all the EcoR1 restriction fragments found on agarose gel as compared to commercially available molecular weight DNA markers. The relative locations of all the clones are established by whether the clones are positive or negative to all the PCR primer sets tested. Because the entirety of the clones were not sequenced, the extent of overlap or gaps (if any) present in the clones has not been characterized. However, the clones are known to contain sequences in common to other clones within the BCR probe set. The total size of the BCR contig is approximately 500 kb.

ABL1

The ABL1 dual fusion probe set consists of 1 BAC, 1 P1, 4 PAC and 1 YAC clone as shown in Table 2, (FIG. 8).

The ABL1 region contains 3 segments of published GenBank sequences: HSALBGR1, 35,692 bp, covering the 5' ABL1 exon 1b and part of intron 1b, HSABLGR2, 59,012 bp containing portions of intron 1b and HSABLGR3, 84,539 bp extending from the end of intron 1b to the end of exon 10 and poly A region. The intron 1b is about 200 kb in length.

The initial screening was done in a similar way to screening for five BCR probes. Three primer sets were synthesized, ABL1 a/b, ABL1 c/d, and ABL1 e/f. ABL1 a/b is located >2000 bp in from the 5' end of the HSALBGR1 sequence, see table 2, FIG. 8. ABL1 c/d is ~79,000 bp in from the 5' end of the HSABLGR3 sequence, and ABL1 e/f is located ~31,000 bp in from the 5' end of HSABLGR2 sequence. The ABL1 a-f primers were used to screen a P1 library directly by PCR and the amplified fragments from these primers were used to screen a PAC library by DNA hybridization. Several positive P1 and PAC clones were identified.

The P1 clone OC3001 was obtained from PCR screening using primers ABL1 a/b. The clone covers a small segment of the HSABLGR1 sequence and extends further in the 5' end of ABL1. A new primer set ABL1 5/6 was made after sequencing the end of the OC3001 insert. ABL1 5/6 was used to screen a PAC library and the PAC clone OCA1001 was acquired. The OCA1001 clone contains the most 5' end of the contig. The P1 clone OC3002 was obtained by PCR screening using primers ABL1 e/f. This clone contains most of the HSABLGR1 and HSABLGR2 sequence regions.

PAC clone OCA1002 was obtained by hybridization screening using the pooled amplified fragments generated by the ABL 1 a-f primers. This PAC clone also extends outside the 5' end of ABL1 gene. The end fragments of the insert were sequenced and primer set ABL1 3/4 was made. ABL1 3/4 was used to screen a BAC library. The BAC clone OCA1003 was identified.

YAC clone OCA1004 was obtained from the commercially available library. OCA1004 contains a portion of HSABLGR2 sequence and extends beyond the 3' end of the HSABLGR3 region. The end fragments of OCA1004 were isolated and sequenced. Primer pair ABL1 7/8 was made and used to screen a PAC library. PAC clone OCA1005 was obtained. A new primer set, ABL1 19/20, was synthesized using sequence information obtained from clone OCA1005. Both PAC OCA1006 and OCA1007 were identified by library screening using ABL1 19/20.

The sizes of the inserts of the clones in the ABL1 probe set, except for the YAC, were estimated by summing up EcoR1 restriction fragments visualized on an agarose gel. The size of YAC clone was determined by comparing to

known size standards on a gel. The relative positions of all the clones were determined from using the primer sets developed for screening DNA bands as physical map anchor sites throughout the ABL1 region. The total length of this contig is approximately 600 kb.

The combination of the BCR and ABL1 probe sets described above defines a dual fusion probe set for t(9;22). It has been designated P5161-DC. The skilled artisan will appreciate that by using these and other techniques known in the art, additional suitable probe sets would be constructed for the ABL1/BCR translocation and for other translocations of interest.

EXAMPLE 2: USING THE PROBE SET FOR CML D-FISH ASSAYS

The P5161-DC probe set was used in standard FISH protocols to devaluate the usefulness of using dual fusion probes (D-FISH) FOR DETECTION. The study of Philadelphia chromosome in a CML clinical population included 37 paired-sets of bone marrow and peripheral blood specimens from 10 patients undergoing treatment for CML, 10 normal peripheral blood specimens, 10 normal bone marrow specimens and four serial dilutions with known percentages of Ph positive nuclei.

Each patient with CML was a participant of the CML National Study Group clinical trial and was randomly receiving treatment with interferon α-2b with or without ara-C. Each patient was known to have cells with a Ph chromosome that produced a typical D-FISH pattern (two fusion signals, two normal signals) for t(9;22)(q34;q11.2). For each patient a paired-set of bone marrow and peripheral blood specimens were collected prior to treatment and at two or more times at approximately 4-month intervals during treatment. Each paired-set of peripheral blood and bone marrow specimens was obtained on the same day except for specimens collected prior to treatment in patients 3 (blood and bone marrow were collected 1 day apart), 5 and 8 (blood and bone marrow were collected 4 days apart).

Uncultured bone marrow and peripheral blood specimens were processed by conventional procedures for cytogenetic and FISH studies. These specimens were stored as fixed pellets at −70° C. in methanol:acetic acid (3:1) until FISH studies could be performed. The D-FISH specimens were prepared by being washed twice with fresh fixative and cells were placed on microscope slides and allowed to air-dry in a CDS-5 cytogenetic drying chamber (Thermotron, Holland, Mich.) adjusted to 50% relative humidity and 25° C. Slides were further dried for 1 hr in a 65° C. oven and then treated with 2× standard saline citrate solution (SSC) (300 mmol/L sodium chloride, 30 mmol/L sodium citrate) for 1 hr at 37° C. Slides were then dehydrated with 70–85–100% cold ethanol (stored at −20° C.) for 2 minutes each, and air-dried.

The FISH hybridization and detection procedure was carried out as follows. Chromosomal DNA (in the form of cells on a slide) was denatured in 70% formamide/2×SSC for 2 min at 70° C. Slides were dehydrated with an ethanol series (70%, 85% and 100%) for 2 min each and air-dried. The probe (Oncor product #P5161-DC) was denatured in a water bath at 70° C. for 5 min. Then 10 μl of stock solution BCR/ABL1 probes were added to each slide, a 22×22 mm coverslip placed on the slide and sealed with rubber cement. Slides were hybridized for 18–20 hrs at 37° C. in a humidified chamber. After the coverslips were removed, slides were washed for 2 min in 0.4×SSC at 70° C., and then in 1× PBD (phosphate-buffered non-ionic detergent) for 2 min. Chromatin was counterstained in blue with 10 μl of 1% solution of 4′,6′-diamidine-2-phenylindole (DAPI) in Vectashield antifade. Representative cells were captured using a computer-based imaging system (Quips XL Genetics Workstation, Vysis, Inc., Downers Grove, Ill.).

Q-cytogenetic studies were performed on each bone marrow specimen by analyzing 25 consecutive G-banded or Q-banded metaphases in which chromosomes 9 and 22 could be observed using the methods of Dewald et al, *Cancer Cytogenet.* 94:59 (1997). Hypermetaphase studies using single fusion probes for BCR and ABL1 (S-FISH) were done on many of these specimens using the methods of Seong et al, *Blood* 86:2343 (1995). D-FISH was performed using the directly labeled P5161-DC probe set to reveal two BCR/ABL1 fusion signals in cells with a t(9;22)(q34;q11.2); one on the abnormal chromosome 9 and the other on the abnormal chromosome 22. The ABL1 (600 kb) probe was directly labeled with Rhodamine Green (green signal) and included several DNA sequences that hybridized to 9 q34 and spanned the 200-Kb breakpoint region of ABL1 including additional normal chromosome sequence on each side of the breakpoint region. The BCR (500 Kb) probe was directly labeled with Texas Red (red signal) and included several DNA sequences that hybridized to 22q11.2 and spanned the common breakpoints in both the major and minor BCR as well as normal chromosome sequences on each side of the BCR gene breakpoint regions.

The specimens were studied in random order and in a blind fashion by two microscopists using strict scoring criteria for D-FISH. Dewald et al, *Blood* 31(9): 3357–3365 (1998). As referred to hereinafter, red BCR signals are referred to as R, green ABL1 signals as G, and BCR/ABL1 fusion signals as F. For scoring purposes, fusion signals were defined as merging or touching R and G signals. The scoring process was limited to normal nuclei with 2 R2 G, and abnormal nuclei with 1R1G2 F or 2R2G1F (one Ph chromosome), and 1R1G3F or 2R2G2F (two Ph chromosomes). For each specimen, each microscopist scored 250 consecutive qualifying interphase nuclei from different areas of the same slide. At the conclusion of the study, the inter-microscopist agreement was sufficient to pool their results on each specimen in subsequent analyses of the data. Thus, the final statistical analyses were based on 500 nuclei per specimen.

The normal range for D-FISH was calculated for peripheral blood specimens collected from 10 patients without any malignant hematologic disorder and for bone marrow specimens collected from 10 normal bone marrow transplant donors. The four serial dilutions were prepared by mixing cells from a normal individual and a Ph positive specimen to create a series of specimens determined by repeated blind studies to contain specified mean percentages of Ph positive nuclei.

The D-FISH results for each patient's specimens from both peripheral blood and bone marrow samples were calculated as the proportion of abnormal cells (number of abnormal cells per 500 scored cells). Since the proportion (p) of abnormal cells among the specimens ranged from 0 to 1 (i.e. 0–100%), a $\sin^1(\sqrt{p})$ transformation was used to stabilize variances and provide a more nearly Gaussian distribution of values. Then, the differences (delta value) between bone marrow and peripheral blood in transformed proportions were computed for each patient's specimens. The proportion (p) of abnormal cells by Q-cytogenetics was also transformed to $\sin^1(\sqrt{p})$.

The delta value for each paired-set of bone marrow and blood specimens was then analyzed using a repeated mea-

sures regression analysis (PROC MIXED in SAS) (19). For purposes of this statistical analysis, the approximate 4 month sampling intervals relative to commencement of therapy was considered a nominal predictor variable and the transformed proportion from Q-cytogenetics was included as a covariate. The within-patient correlation of delta values among respective specimen collection times was specified as an autocorrelation structure depending on the actual number of days between sampling times i.e., smaller correlations between sequential values for longer times between sampling episodes.

The classification scheme for response to therapy was based on Q-cytogenetics and was similar to the Italian Cooperative Group (Italian Cooperative Study Group on Chronic Myeloid Leukemia *New England Journal of Medicine* 30:820 (1994)) i.e., no response, minimal, minor, major and complete remission when 100%, 99–67%, 66–33%, 32–1% and 0% of metaphases are Ph positive, respectively.

Probe Sets in a D-FISH Assay Demonstrate Higher Sensitivity Than Standard Cytogenetic Testing

The goal was to study the effectiveness of the P5161-DC probes using 500 nuclei for each bone marrow and blood specimen. The goal for Q-cytogenetics was to study 25 metaphases from each bone marrow specimen. The goal for hypermetaphase studies was to study 200 metaphases from bone marrow. D-FISH was successful on 37/37 blood specimens and 37/37 bone marrow specimens. Q-cytogenetic was successful in 32/37 bone marrow specimens. Hypermetaphase was successful in 14/24 bone marrow specimens.

Very Low False Positive Rate (<1.0%)

Based on 500 nuclei from each of 10 normal bone marrow specimens, the mean percentage and standard deviation of nuclei with false BCR/ABL1 fusion was 0.1%±0.1 (range 0 to 1 per 500 nuclei). Based on 500 nuclei from each of 10 normal peripheral blood specimens, the mean percentage and standard deviation of nuclei with false BCR/ABL1 fusion was 0.04%±0.08. Based on this data, the upper bound of a one-sided 95% confidence interval for observing 1 to 500 (0.2%) neoplastic cells in either bone marrow or peripheral blood was calculated using the binomial distribution. For both bone marrow and peripheral blood, this calculation implied a cutoff greater than 4/500 (>0.8%) nuclei with BCR/ABL1 fusion to classify any specimen as abnormal.

Abnormal Reference Range for D-FISH in Untreated CML

The results of D-FISH for specimens from seven patients (nos. 2–7, 9) that were collected prior to treatment and that were not mosaic by Q-cytogenetic studies were used to establish an abnormal reference range. These specimens generally represent patients with untreated CML in clinical practice. Among these seven specimens, the mean percentage of abnormal cells was 97.6%±1.38 (range 95.4 to 99.0) for bone marrow, and 86.1%±13.59 (range 61.6 to 98.5) for blood.

Serial Dilutions

The observed percentage of neoplastic cells in each of the four serial dilution specimens was 97.6, 49.2, 8.2 and 1.8. The expected mean percentage of neoplastic cells in these specimens was 98.2, 49.1, 10.7, and 2.8, respectively. The difference between observed and expected values for each of these specimens was 0.6%, 0.1%, 2.5% and 1.0%, respectively.

Results of Using the Probe Set in a D-FISH Assay With Clinical Specimens

Results for Q-cytogenetic studies for bone marrow, and D-FISH for bone marrow and blood for each patient specimen are shown in FIG. 3. Based on Q-cytogenetics, three patients (nos. 4, 5 and 6) achieved a complete cytogenetic remission, one patient (no. 3) briefly achieved a major response and the rest of the patients were classified as minimal, minor or non-responders.

Each bone marrow specimen that had any abnormal metaphases by Q-cytogenetics was also abnormal for interphase nuclei by D-FISH in blood and bone marrow. Six specimens from three patients (nos. 4, 5 and 6) had only normal metaphases by Q-cytogenetics. For patient 6, D-FISH results were abnormal at 357 days in both bone marrow (4.8% abnormal nuclei) and blood (3.0% abnormal nuclei). For patient 5 at 262 days, the peripheral blood was marginally abnormal (1.0% abnormal nuclei) but bone marrow was within normal limits (0.6% abnormal nuclei). Each of the remaining four specimens with only normal metaphases by Q-cytogenetics were within normal limits for D-FISH in both bone marrow and blood.

Detection of Minimal Residual Disease States and Tracking Response to Therapy using the Probe Sets in FISH

Additional studies on the paired-sets of bone marrow and blood specimens that were normal by Q-cytogenetics and D-FISH were done to look for minimal residual disease. In a blind study, D-FISH was used to score 6,000 nuclei from four of the bone marrow specimens and five of the peripheral blood specimens in this series (Table 3, FIG. 9), and 3 blood and bone marrow specimens from normal individuals. In a separate study, the normal range for D-FISH for 6,000 nuclei was calculated to be <0.079%. Based on this cutoff, each of the normal blood and bone marrow specimens was correctly classified as normal. Three of the four patient bone marrow specimens and each of the patient peripheral blood specimens had minimal residual disease. It was not possible to do further studies on bone marrow no. 5 from patient 4 as this specimen had no leftover cells. The paired-blood specimen for this sampling time was in the abnormal range for D-FISH when 6,000 nuclei were studied and the bone marrow and one Ph positive metaphase among 169 metaphases that were examined by hypermetaphase FISH studies.

The actual proportions of neoplastic cells from bone marrow specimens were plotted against the corresponding proportions from peripheral blood samples (FIG. 2). The results imply that the proportion of abnormal cells from bone marrow specimens was typically greater (above y=x line) than for peripheral blood.

For D-FISH, the mean 4 month inter-sample differences in percentage of abnormal nuclei between paired-sets of bone marrow and peripheral blood were not statistically different (p>0.3)(Table 4, FIG. 10). The deltas for D-FISH for peripheral blood were associated (p<0.05) with the transformed proportion of abnormal cells based on Q-cytogenetics of the paired bone marrow specimen. This is important because Q-cytogenetics of bone marrow is widely recognized as the "gold standard" for monitoring response to interferon therapy.

Based on these results, an additional regression analysis was done to develop a model for estimating the proportion of abnormal cells that would be obtained from bone marrow specimens using D-FISH results from peripheral blood samples. This is regression analysis of the data displayed in

FIG. 4, but used the transformed values of the proportions (FIG. 3). In FIG. 5, the dashed lines represent an approximate 95% confidence interval for a new predicted observation given a (new) peripheral blood value (prediction interval). This analysis indicated a significant ($p<0.001$) linear relationship and yields the following equation for estimating the proportion of abnormal cells in bone marrow

specimens (P_{BM}), $\hat{P}_{BM}=[\sin\{0.1494+1.0324*\sin^{1}(\sqrt{P_{PB}})\}]^2$,

where P_{PB} is the proportion of abnormal cells based on D-FISH results in peripheral blood samples. This relationship is displayed in FIG. 6, and the numeric results for several choices of P_{PB} is listed in Table 5.

Discussion

The 4-month inter-sample changes in percentage of neoplastic nuclei in blood agreed closely with the corresponding intersample changes in percentage of neoplastic metaphases and nuclei in bone marrow over the course of interferon α-2b therapy. The reduction in percentage of Ph positive metaphases correlates with a prolonged chronic phase and increased survival in CML and the results of D-FISH on blood correlates with Q-cytogenetics. This demonstrates that using probes according to the present invention in a FISH assay is efficacious to test periodic peripheral blood specimens from patients with CML to monitor the effectiveness of interferon therapy. The analysis of 500 nuclei with the P5161-DC probe set in a D-FISH in bone marrow and peripheral blood detects <1% disease and is at least as sensitive as Q-cytogenetics. Thus, D-FISH analyses of interphase nuclei using probe constructs according to the present invention could substitute for Q-cytogenetics for purposes of monitoring response to therapy for CML. By analyzing 6,000 nuclei in specimens that were normal by Q-cytogenetics and by D-FISH based on analysis of 500 nuclei revealed evidence of residual disease was found (Table 4, FIG. 10). Thus, the methods and probe sets of the invention have the potential to detect very low levels of minimal disease in both blood and bone marrow.

In one other experiment that compares the results of FISH studies of paired-sets of bone marrow and peripheral blood to monitor therapy in CML, Muhlmann et al, *Genes, Chromosomes and Cancer* 21:90 (1998) used S-FISH to study 49 peripheral blood smears and 30 bone marrow specimens from 36 patients in chronic phase CML at different stages of cytogenetic remission. This experiment establishes that one can use whole blood as a comparative measure for events in the bone marrow.

The present invention precisely predicts the percentage of neoplastic nuclei in bone marrow based on data from blood. This should allow one to use blood to monitor therapy in clinical practice. The results presented in the present specification indicate that it is best to assess response to therapy based on changes in percentage of neoplastic nuclei using the same tissue over time. In other words, to compare D-FISH results among blood studies or among bone marrow studies, but not between blood and bone marrow studies. This is important because the percentage of abnormal nuclei in blood and bone marrow differs in most patients at most times before and after therapy (FIG. 4).

The results show a strong correlation between changes in the percentage of Ph positive metaphases by Q-cytogenetic studies over the course of therapy and changes in the percentage of interphase nuclei with BCR/ABL1 fusion in both blood and bone marrow. D-FISH using the probes of the invention was also useful to identify residual disease in both bone marrow and peripheral blood specimens for patients in complete cytogenetic remission. For patients on therapy, D-FISH could then be performed on peripheral blood at periodic intervals to assess the effectiveness of therapy. Consequently, bone marrow would not need to be collected to monitor therapy as frequently or at all as it is in current practice.

More details regarding scoring and correlation to clinical patients may be found in Dewald et al, Blood 31(9): 3357–3365 (1998).

EXAMPLE 3: CONSTRUCTION OF AML1/ETO DUAL FUSION PROBES

The AML1/ETO also called MTG8/CDR dual probes were assembled using the same method as in EXAMPLE 1 above. The highlights being illustrated below. The breakpoints are known to be clustered, Miyoshi et al (1991), Erickson et al (1992), Shimizu et al (1992), and Tighe et al (1993). The translocation has traditionally been detected using reverse transcriptase mediated polymerase chain reaction.

Two overlapping YACs, 902G10 and 903A9 were isolated from a total human library using an ETO cDNA probe. The YACs spanned the entire 8q22 breakpoint region. YAC C14B2 is predominantly located proximal to the 21q22 breakpoint region. YAC 925E1 was obtained from a total human library and includes a region located immediately distal to the breakpoint region.

The YAC DNAs 902G10 and 903A9 were labeled by nick translation with digoxigenin and C14B2 and 925E1 were labeled with biotin. FITC was used to detect biotin labeled probe molecules and rhodamine was used to detect digoxigenin labeled probe molecules using detection kits (Oncor, Inc.)

EXAMPLE 4: D-FISH FOR THE AML1/ETO TRANSLOCATION

The methods of Example 2 were repeated using the probe set of Example 3 with AML cell line Kasumi-1, lymphoblastoid cell line GM09948, bone marrow. Excellent results were obtained either two clear fusion signals being seen in a large percentage of cells. Details may be seen in Paskulin et al, Genes, Chromosomes & Cancer 21:144–151 (1998). The method of Example 2 is also performed on peripheral blood cells and correlated to the bone marrow data.

References cited herein are hereby incorporated by reference, and are listed below for convenience:

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

What is claimed is:

1. A DNA probe set, said probe set comprising a first probe set and a second probe set,

said first probe set being sufficient in length and substantially complementary to an entire breakpoint region of a first DNA and nucleotides on both sides of the breakpoint region but less than an entire chromosome such that said first probe set will hybridize to both sides of the breakpoint region regardless of whether the first DNA has been broken in the breakpoint region and either end fused to another DNA, and

said second probe set being sufficient in length and substantially complementary to an entire breakpoint region of a second DNA and nucleotides on both sides of the breakpoint region but less than an entire chromosome such that said second probe set will hybridize to both sides of the breakpoint region regardless of whether the second DNA has been broken in the breakpoint region and either end fused to another DNA.

2. The probe set of claim **1**, wherein said probes are detectably labelled.

3. The probe set of claim **2**, wherein said first DNA is part of the ABL1 gene on chromosome 9 and the second DNA is part of the BCR gene on chromosome 22.

4. The probe set of claim **2**, wherein said first DNA is part of the AML1 gene on chromosome 21 and the second DNA is part of the ETO gene on chromosome 8.

5. A diagnostic kit for detecting a structural abnormality caused by chromosomal breakage and rearrangement containing a reagent comprising at least one probe set of the probe set according to claim 1, and a container containing said reagent.

6. A diagnostic kit according to claim **5** further comprising at least two containers, wherein a first container contains a reagent comprising said first probe set and a second container contains a reagent comprising said second probe set.

7. A diagnostic kit according to claim **6** wherein said reagent comprises said first and said second probe set.

8. A DNA probe set, said probe set comprising a first probe set and a second probe set,

said first probe set being sufficient in length and substantially complementary to an entire breakpoint region of a first DNA and nucleotides on both sides of the breakpoint region but less than an entire chromosome such that said first probe set will hybridize to both sides of the breakpoint region regardless of whether a second DNA from a region other than the breakpoint region has been inserted in the breakpoint region, and

said second probe set being sufficient in length and substantially complementary to a 3' end and a 5' end of a second DNA but less than an entire chromosome such that said second probe set will hybridize to both ends of the second DNA regardless of whether the second DNA is inserted in the first DNA.

9. The probe set of claim **8**, wherein said probes are detectably labelled.

10. A DNA probe set, said probe set comprising a first probe set and a second probe set,

said first probe set being sufficient in length and substantially complementary to nucleotides on both sides of the breakpoint region of a first DNA but less than an entire chromosome such that said first probe set will hybridize to both sides of the breakpoint region regardless of whether the first DNA has been broken in the breakpoint region and either end fused to another DNA, and

said second probe set being sufficient in length and substantially complementary to nucleotides on both sides of the breakpoint region of a second DNA but less than an entire chromosome such that said second probe set will hybridize to both sides of the breakpoint region regardless of whether the second DNA has been broken in the breakpoint region and either end fused to another DNA.

11. The probe set of claim **10**, wherein said probes are detectably labeled.

12. The probe set of claim **11**, wherein said first DNA is part of the ABL1 gene on chromosome 9 and the second DNA is part of the BCR gene on chromosome 22.

13. The probe set of claim **11**, wherein said first DNA is part of the AML1 gene on chromosome 21 and the second DNA is part of the ETO gene on chromosome 8.

14. A diagnostic kit for detecting a structural abnormality caused by chromosomal breakage and rearrangement containing a reagent comprising at least one probe set of the probe set according to claim **10**, and a container containing said reagent.

15. A diagnostic kit according to claim **14** further comprising at least two containers, wherein a first container contains a reagent comprising said first probe set and a second container contains a reagent comprising said second probe set.

16. A diagnostic kit according to claim **15** wherein said reagent comprises said first and said second probe sets.

17. A diagnostic kit for detecting a structural abnormality caused by chromosomal breakage and rearrangement containing a reagent comprising at least one probe set of the probe set according to claim **8**, and a container containing said reagent.

18. A diagnostic kit according to claim **17** further comprising at least two containers, wherein a first container contains a reagent comprising said first probe set and a second container contains a reagent comprising said second probe set.

19. A diagnostic kit according to claim **18** wherein said reagent comprises said first and said second probe sets.

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Civil Cover Sheet

This automated JS-44 conforms generally to the manual JS-44 approved by the Judicial Conference of the United States in September 1974. The data is required for the use of the Clerk of Court for the purpose of initiating the civil docket sheet. The information contained herein neither replaces nor supplements the filing and service of pleadings or other papers as required by law. This form is authorized for use only in the Northern District of Illinois.

Plaintiff(s): Ventana Medical Systems, Inc.

County of Residence:

Plaintiff's Atty: David Berten
Competition Law Group, LLC
120 South State Street, Suite 300, Chicago ,IL 60603
(312) 629-1904

Defendant(s):Vysis, Inc. and Abbott Laboratories ARCH Development Corporation

County of Residence:

Defendant's Atty:

03C 4870

JUDGE LINDBERG

MAGISTRATE SIDNEY I. SCHENKIER

II. Basis of Jurisdiction: **3. Federal Question (U.S. not a party)**

III. Citizenship of Principal Parties **(Diversity Cases Only)**

Plaintiff:- **N/A**
Defendant:- **N/A**

IV. Origin : **1. Original Proceeding**

V. Nature of Suit: **830 Patent**

VI.Cause of Action: **Patent Infringement (35 U.S.C. §271)**

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CLERK
U.S. DISTRICT COURT

VII. Requested in Complaint

Class Action: **No**
Dollar Demand: **Unspecified**

1-2

Jury Demand: **Yes**

VIII. This case **IS** a refiling of a previously dismissed case. Case number **D.AZ-01-CV-593** by Judge **Wiliam D. Browning**

Signature: [signature]

Date: 15 July 2003

If any of this information is incorrect, please go back to the Civil Cover Sheet Input form using the *Back* button in your browser and change it. Once correct, print this form, sign and date it and submit it with your new civil action. **Note: You may need to adjust the font size in your browser display to make the form print properly.** **Revised: 06/28/00**

UNITED STATES DISTRICT COURT
NORTHERN DISTRICT OF ILLINOIS

EASTERN DIVISION

DOCKETED
JUL 1 6 2003

In the Matter of

Ventana Medical Systems, Inc.
v.
Vysis, Inc. and Abbott Laboratories
ARCH Development Corporation

03C 4870
Case Number:

APPEARANCES ARE HEREBY FILED BY THE UNDERSIGNED AS ATTORNEY(S) FOR:

JUDGE LINDBERG

Ventana Medical Systems, Inc., Plaintiff

MAGISTRATE SIDNEY I. SCHENKIER

(A)		(B)	
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FIRM Competition Law Group, LLC		FIRM Same as A	
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IDENTIFICATION NUMBER (SEE ITEM 4 ON REVERSE) 6200898		IDENTIFICATION NUMBER (SEE ITEM 4 ON REVERSE) 6201080	
MEMBER OF TRIAL BAR?	YES ☑ NO ☐	MEMBER OF TRIAL BAR?	YES ☑ NO ☐
TRIAL ATTORNEY?	YES ☑ NO ☐	TRIAL ATTORNEY?	YES ☑ NO ☐
		DESIGNATED AS LOCAL COUNSEL?	YES ☐ NO ☑

(C)		(D)	
SIGNATURE		SIGNATURE	
NAME Greg Smith		NAME Kelly Eberspecher	
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STREET ADDRESS		STREET ADDRESS	
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MEMBER OF TRIAL BAR?	YES ☑ NO ☐	MEMBER OF TRIAL BAR?	YES ☐ NO ☑
TRIAL ATTORNEY?	YES ☑ NO ☐	TRIAL ATTORNEY?	YES ☑ NO ☐
DESIGNATED AS LOCAL COUNSEL?	YES ☐ NO ☑	DESIGNATED AS LOCAL COUNSEL?	YES ☐ NO ☑

FILED-ED4
03 JUL 15 PM 12:21
CLERK U.S. DISTRICT COURT

UNITED STATES DISTRICT COURT
NORTHERN DISTRICT OF ILLINOIS
EASTERN DIVISION

DOCKETED
JUL 16 2003

In the Matter of

Ventana Medical Systems, Inc.
v.
Vysis, Inc. and Abbott Laboratories
ARCH Development Corporation

Case Number: 03C 4870
JUDGE LINDBERG

APPEARANCES ARE HEREBY FILED BY THE UNDERSIGNED AS ATTORNEY(S) FOR:
MAGISTRATE SIDNEY I. SCHENKIER

Ventana Medical Systems, Inc., Plaintiff

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SIGNATURE [signature]		SIGNATURE	
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MEMBER OF TRIAL BAR?	YES ☐ NO ☑	MEMBER OF TRIAL BAR?	YES ☐ NO ☐
TRIAL ATTORNEY?	YES ☐ NO ☑	TRIAL ATTORNEY?	YES ☐ NO ☐
		DESIGNATED AS LOCAL COUNSEL?	YES ☐ NO ☐

(C)		(D)	
SIGNATURE		SIGNATURE	
NAME		NAME	
FIRM		FIRM	
STREET ADDRESS		STREET ADDRESS	
CITY/STATE/ZIP		CITY/STATE/ZIP	
TELEPHONE NUMBER	FAX NUMBER	TELEPHONE NUMBER	FAX NUMBER
E-MAIL ADDRESS		E-MAIL ADDRESS	
IDENTIFICATION NUMBER (SEE ITEM 4 ON REVERSE)		IDENTIFICATION NUMBER (SEE ITEM 4 ON REVERSE)	
MEMBER OF TRIAL BAR?	YES ☐ NO ☐	MEMBER OF TRIAL BAR?	YES ☐ NO ☐
TRIAL ATTORNEY?	YES ☐ NO ☐	TRIAL ATTORNEY?	YES ☐ NO ☐
DESIGNATED AS LOCAL COUNSEL?	YES ☐ NO ☐	DESIGNATED AS LOCAL COUNSEL?	YES ☐ NO ☐

FILED-ED4
03 JUL 15 PM 12:21
U.S. DISTRICT COURT

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