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(54) **RANDOM GENE UNIDIRECTIONAL
ANTISENSE LIBRARY**

Publication Classification

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(57) **ABSTRACT**

The present invention provides a high-throughput system for functional genomics using a random gene unidirectional antisense library comprising LC-antisense compounds. The antisense compounds were specific and effective for the elimination of target mRNA. Thus, the system of the present invention may be effectively used as temporary knock-down system to unveil functions of genes critical for diseases. The system of the present invention can be adapted not only for functional genomics but also for effectively validating target for antisense or other molecular therapeutics against various malignancies, infections, and other diseases by blocking specific genes involved in the disease.

(73) Assignee: **WELGENE, INC.**

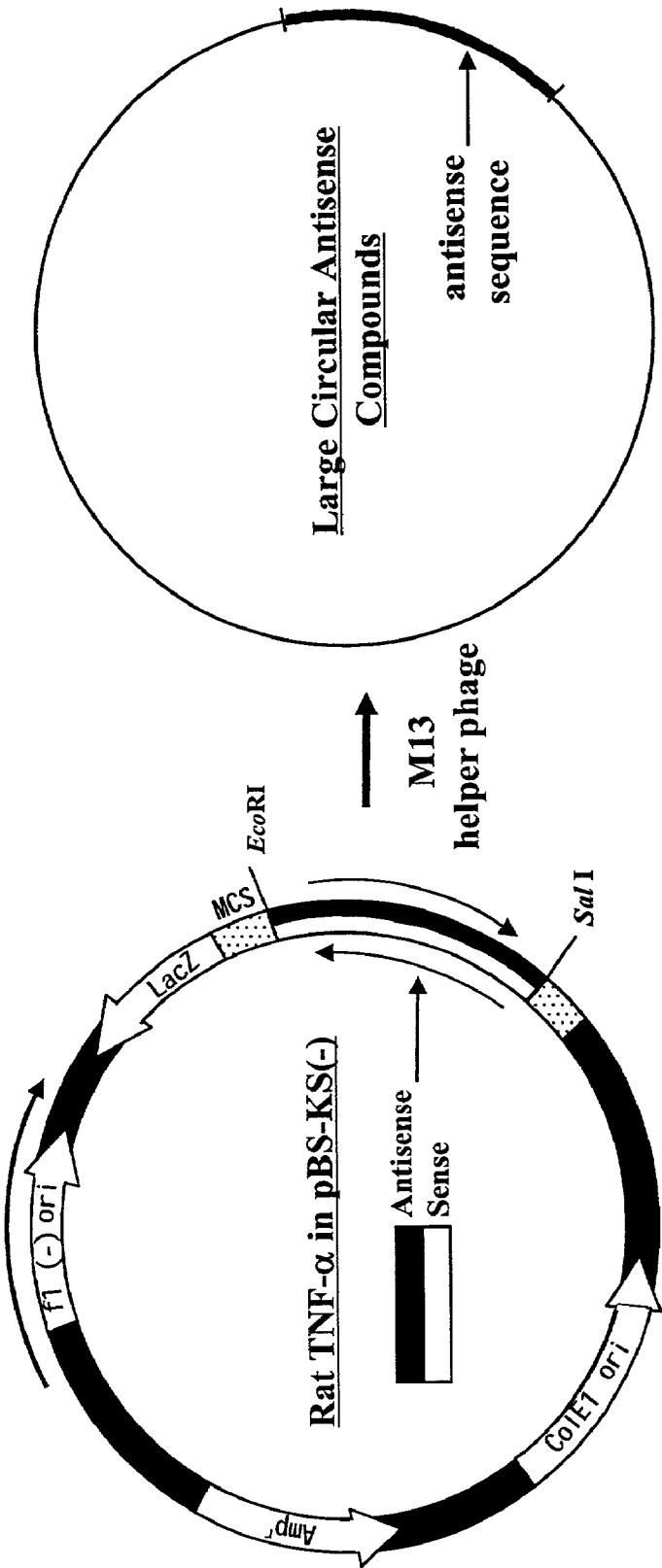
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(22) Filed: **Mar. 8, 2002**

(30) **Foreign Application Priority Data**

Mar. 8, 2001 (KR) 2001-12061

Fig. 1



Single-strand rescue by helper bacteriophages

Fig. 2

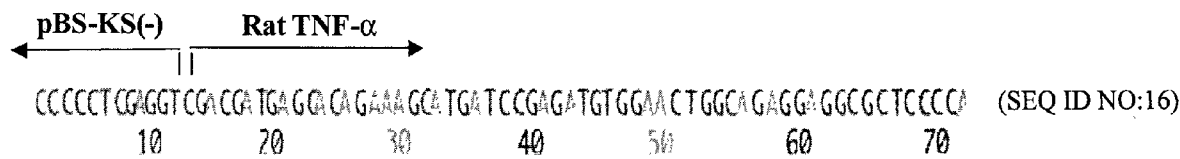
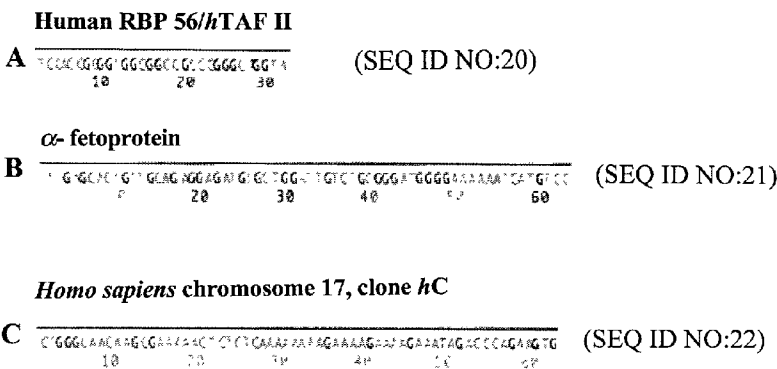


Fig. 12



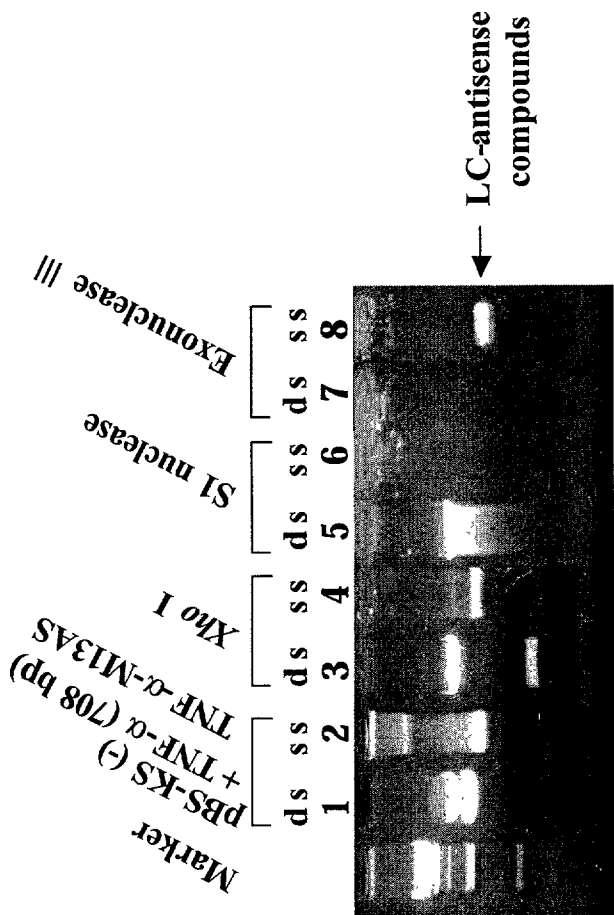


Fig. 3A

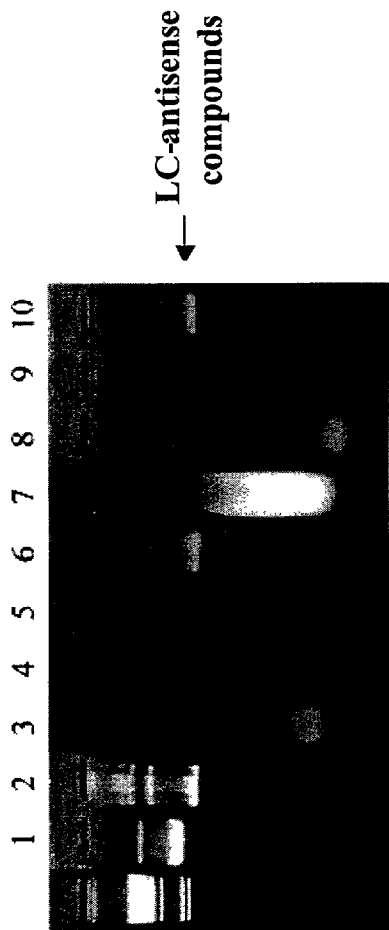


Fig. 3B

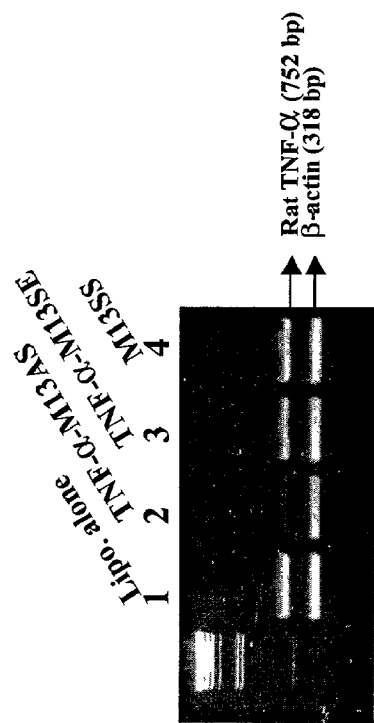


Fig. 4A

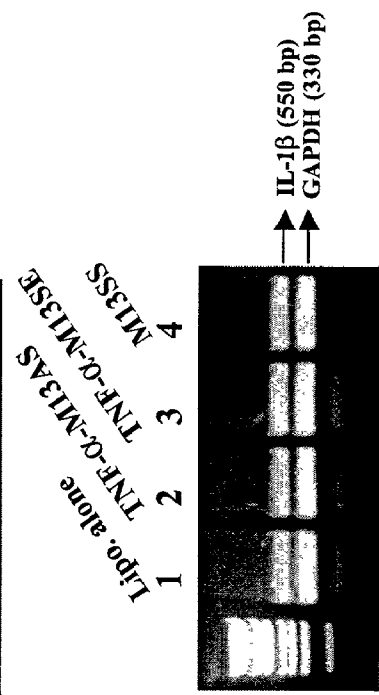


Fig. 4B

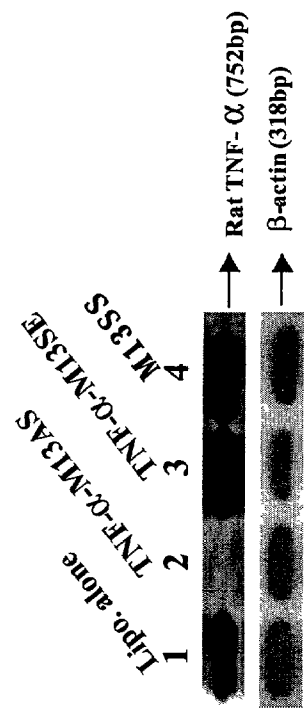
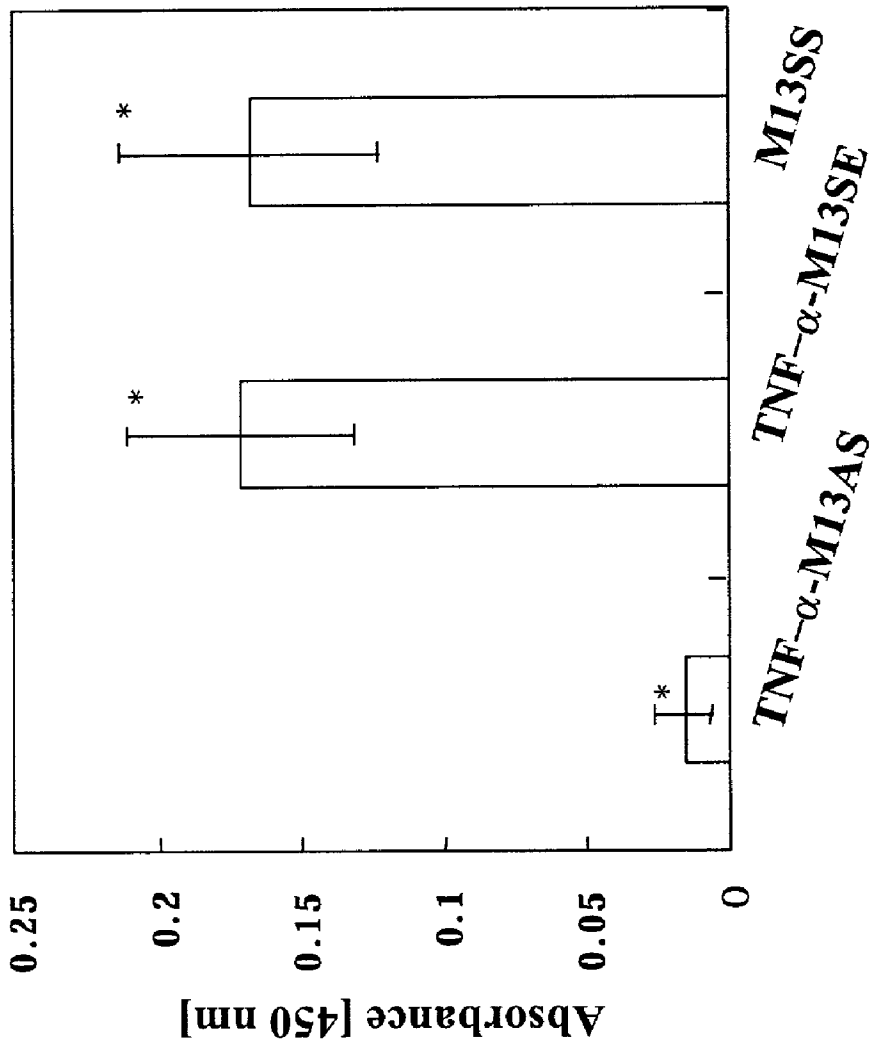


Fig. 4C

Fig. 5



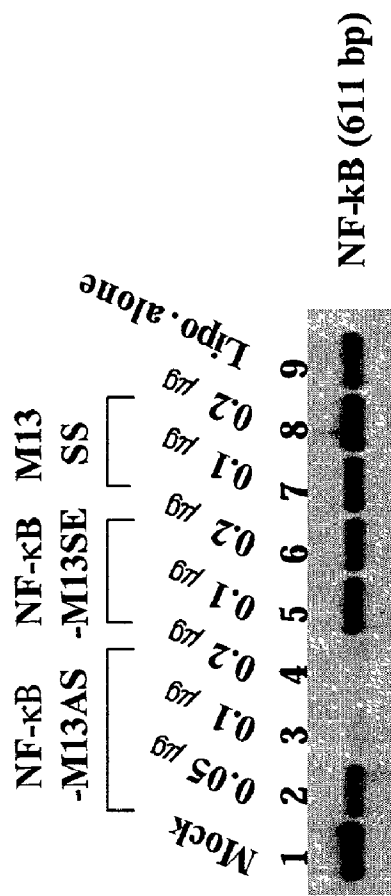
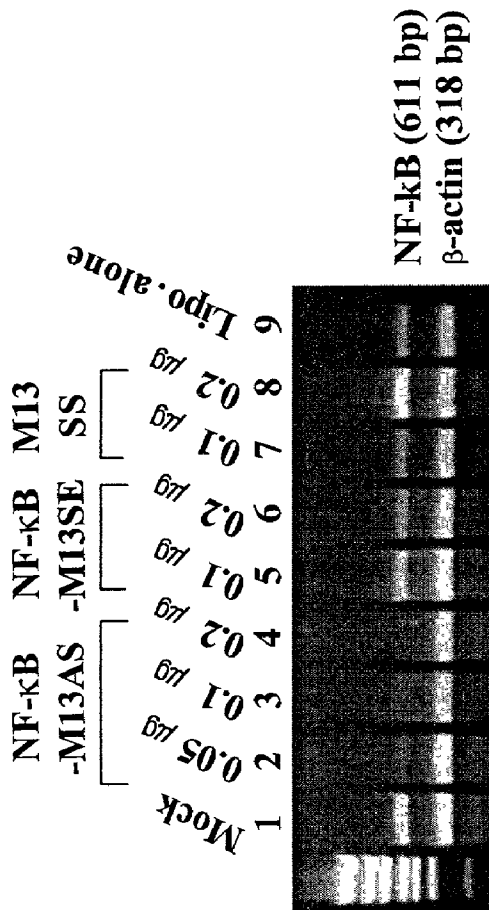


Fig. 7

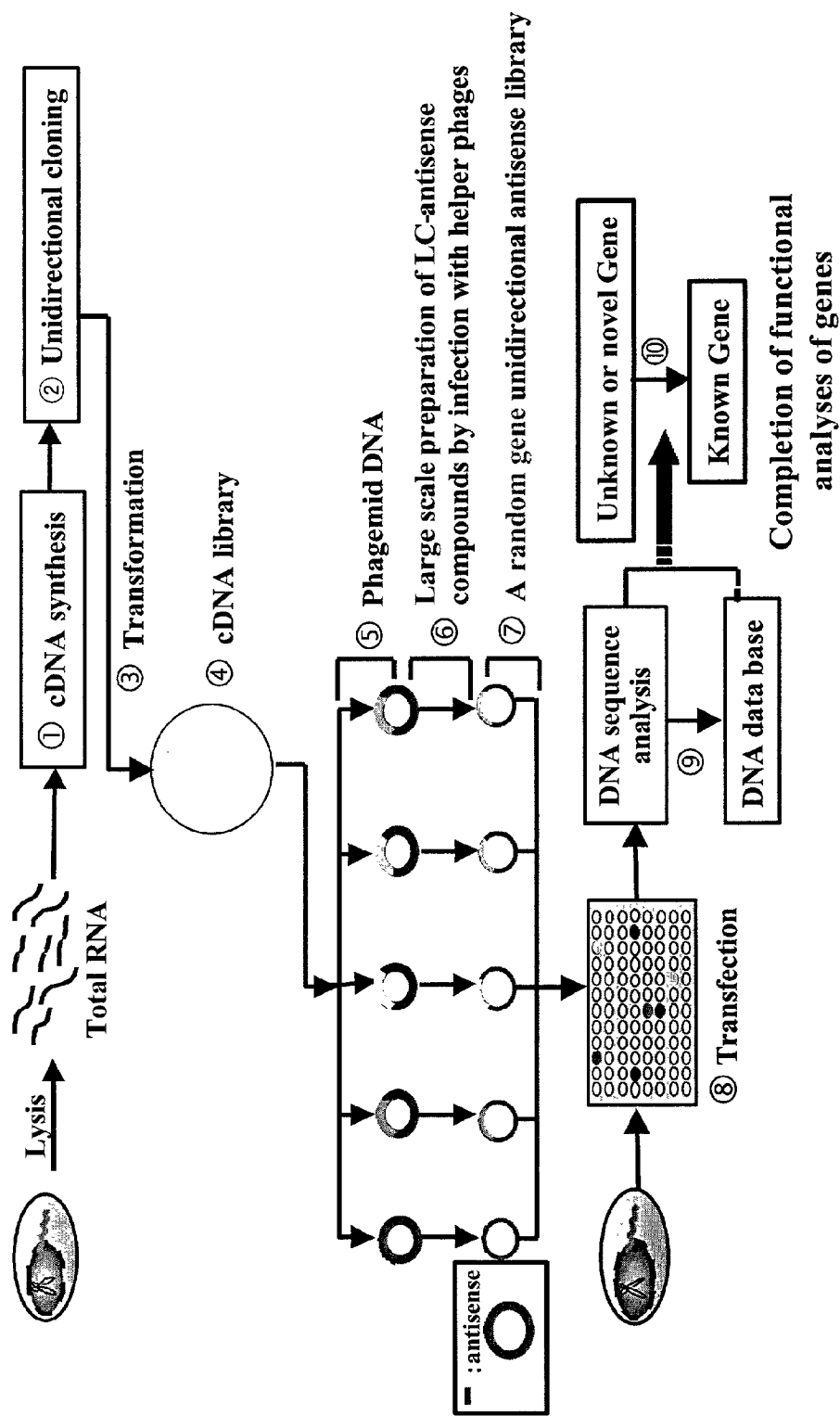


Fig. 8

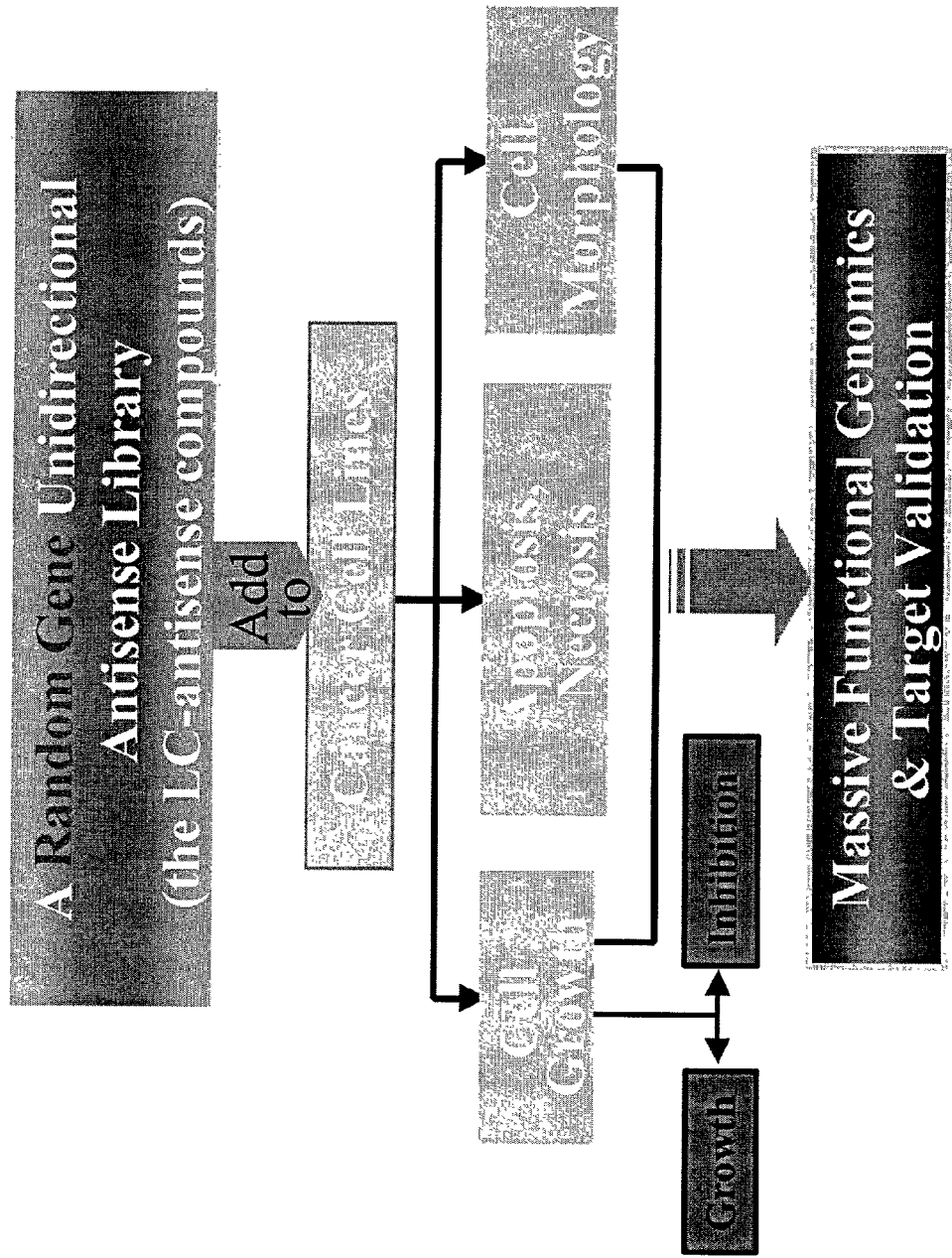


Fig. 9

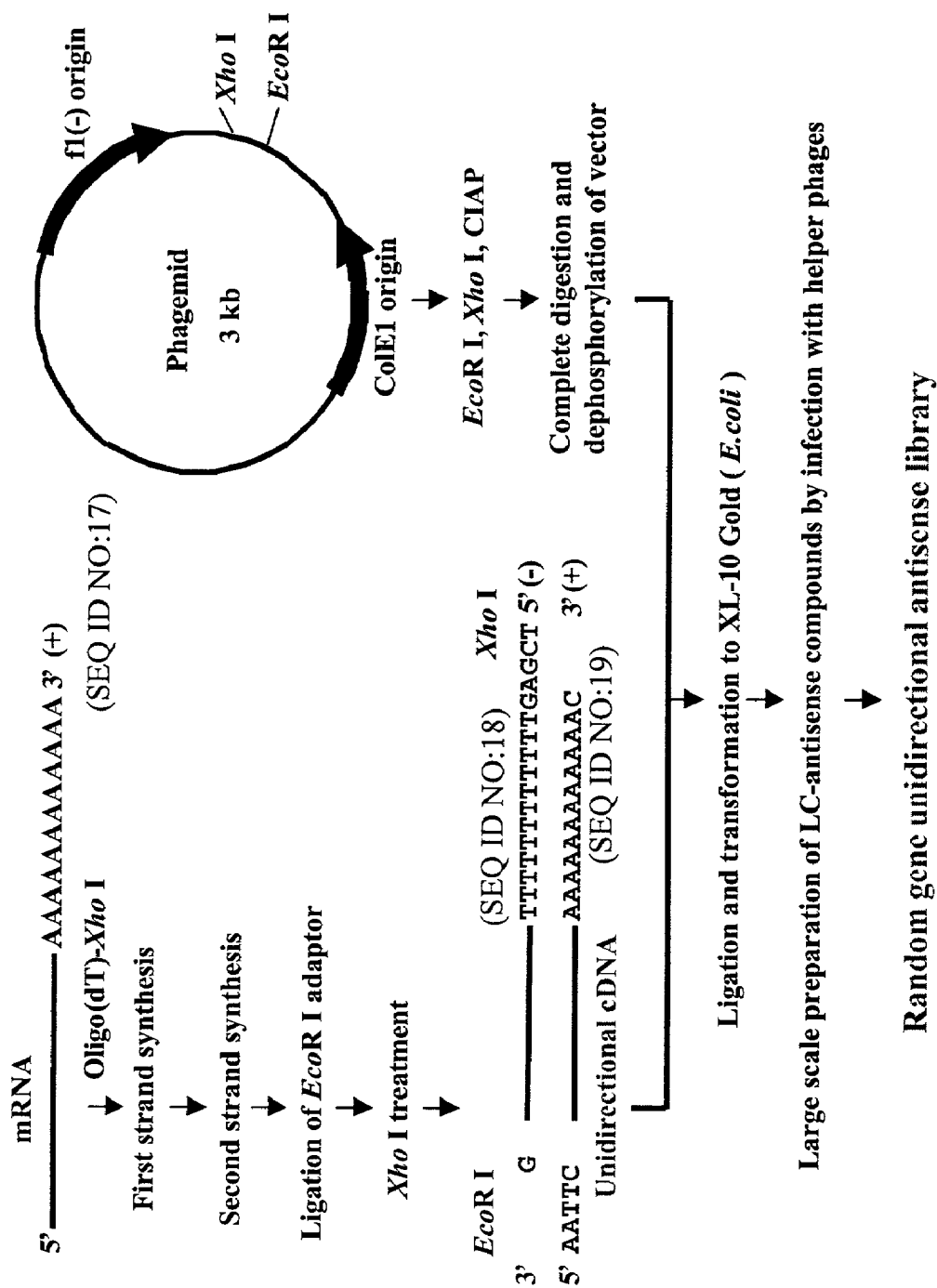


Fig. 10

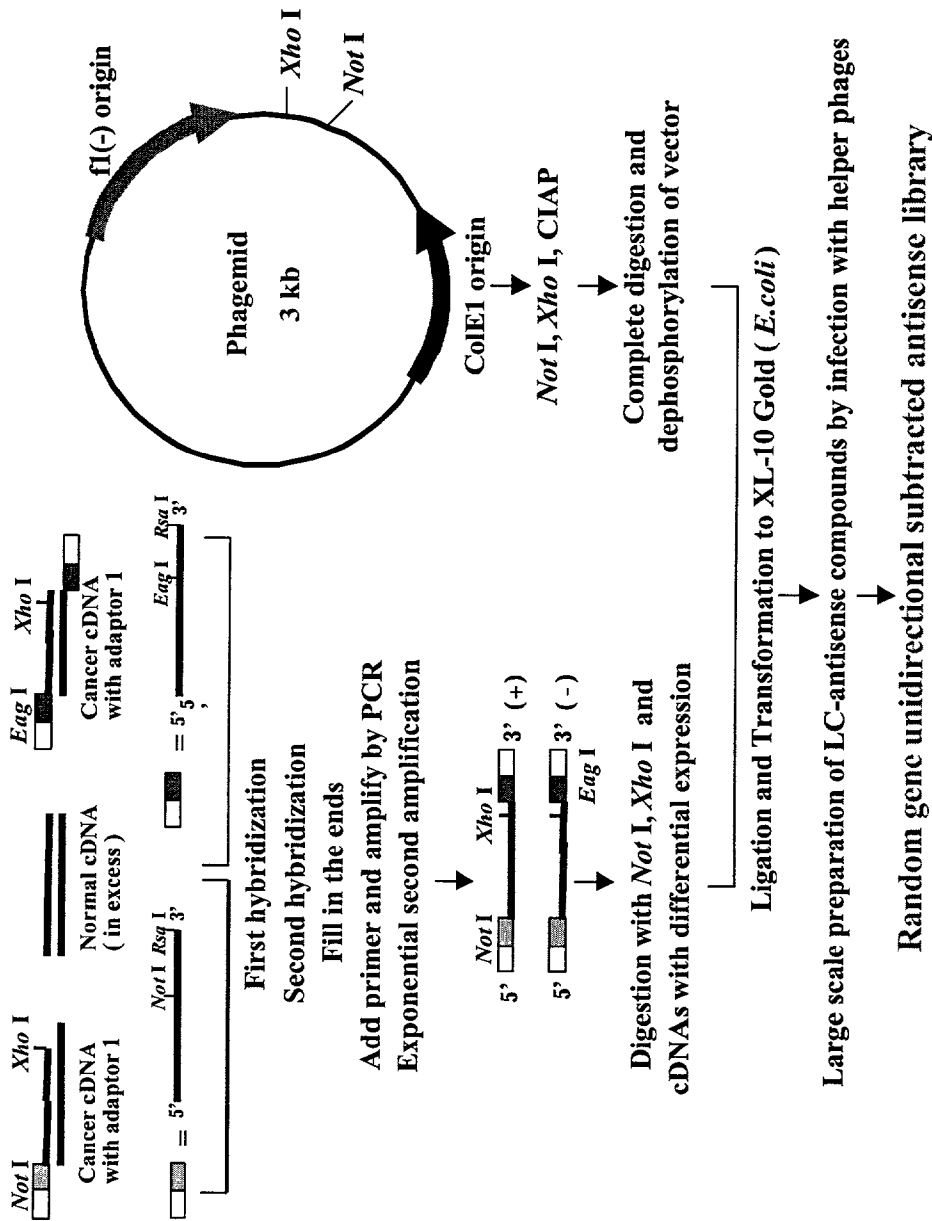


Fig. 11

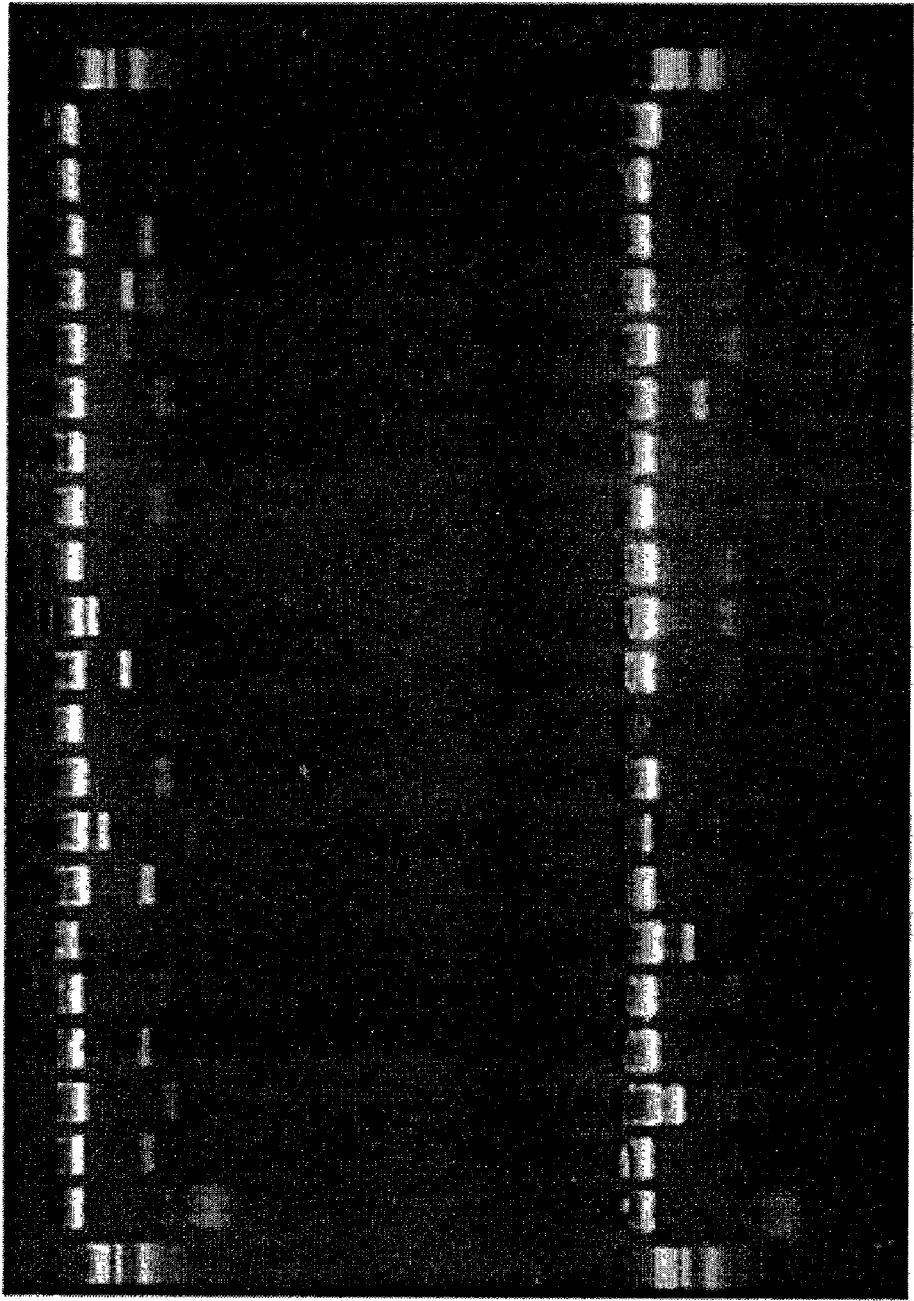
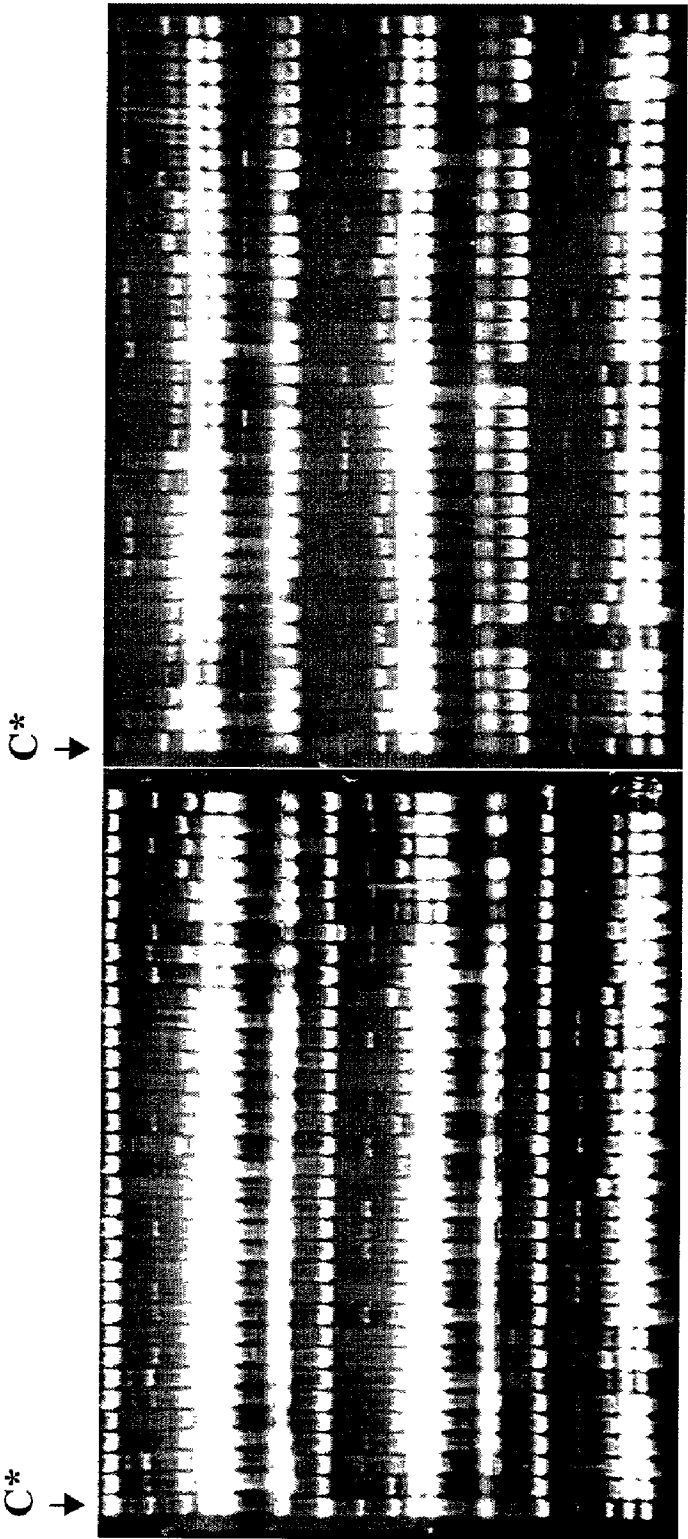


Fig. 13



C* : Recombinant phagemid DNA with a cDNA fragment of 500 bp of cDNA used as a control for selection

Fig. 14

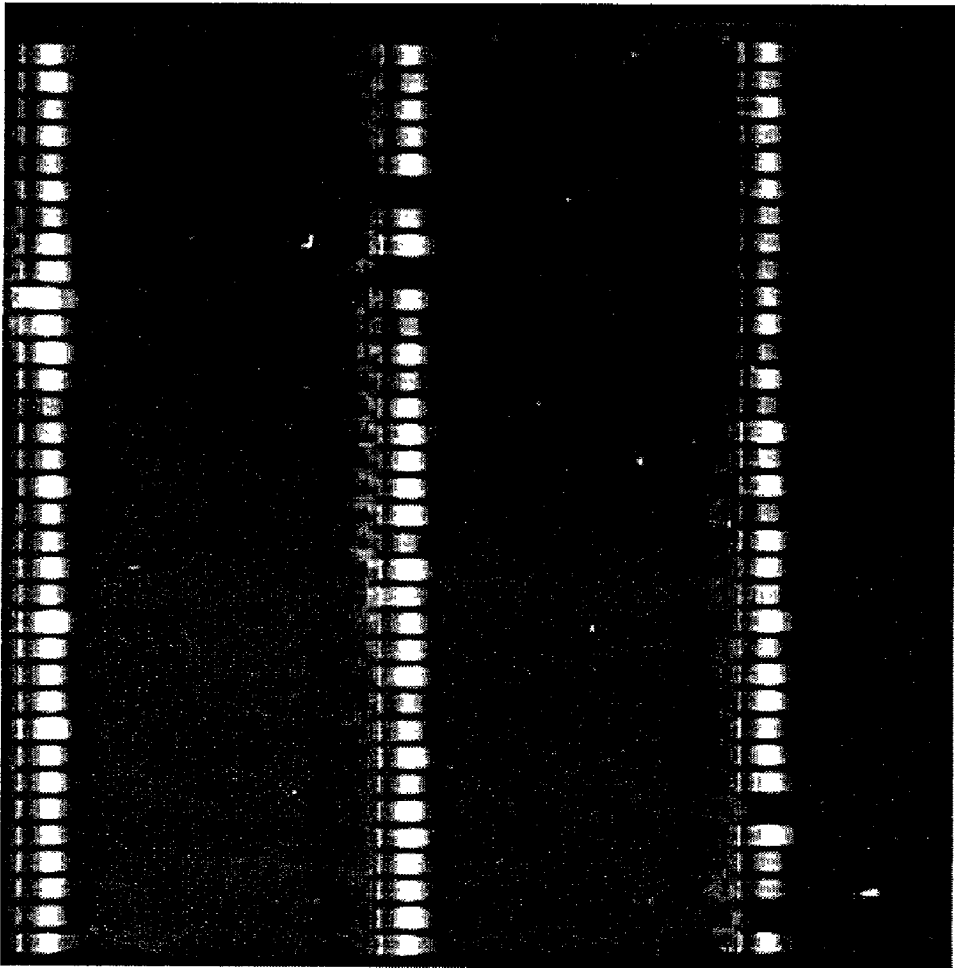


Fig. 15
HepG2 cell line

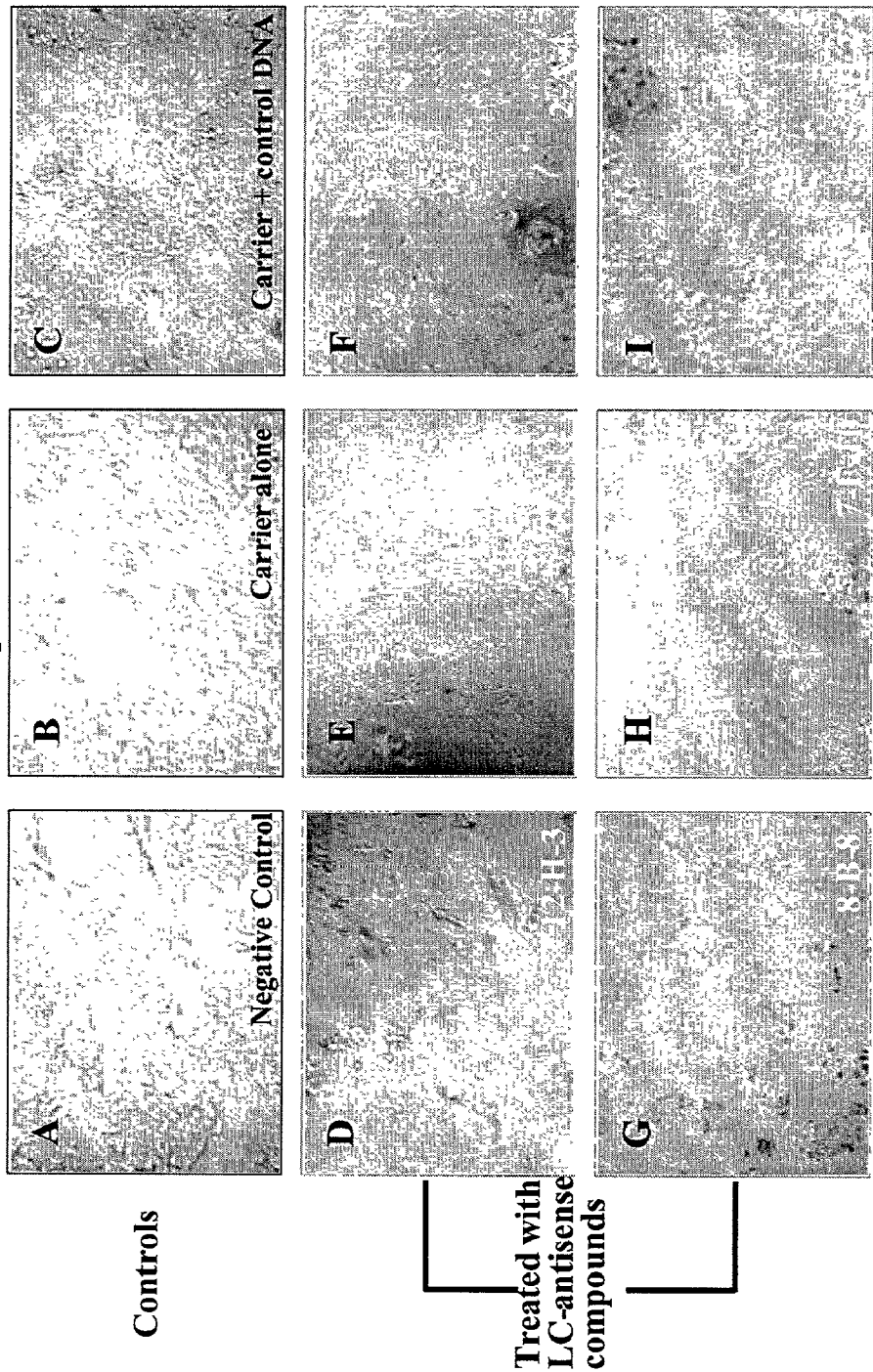


Fig. 16

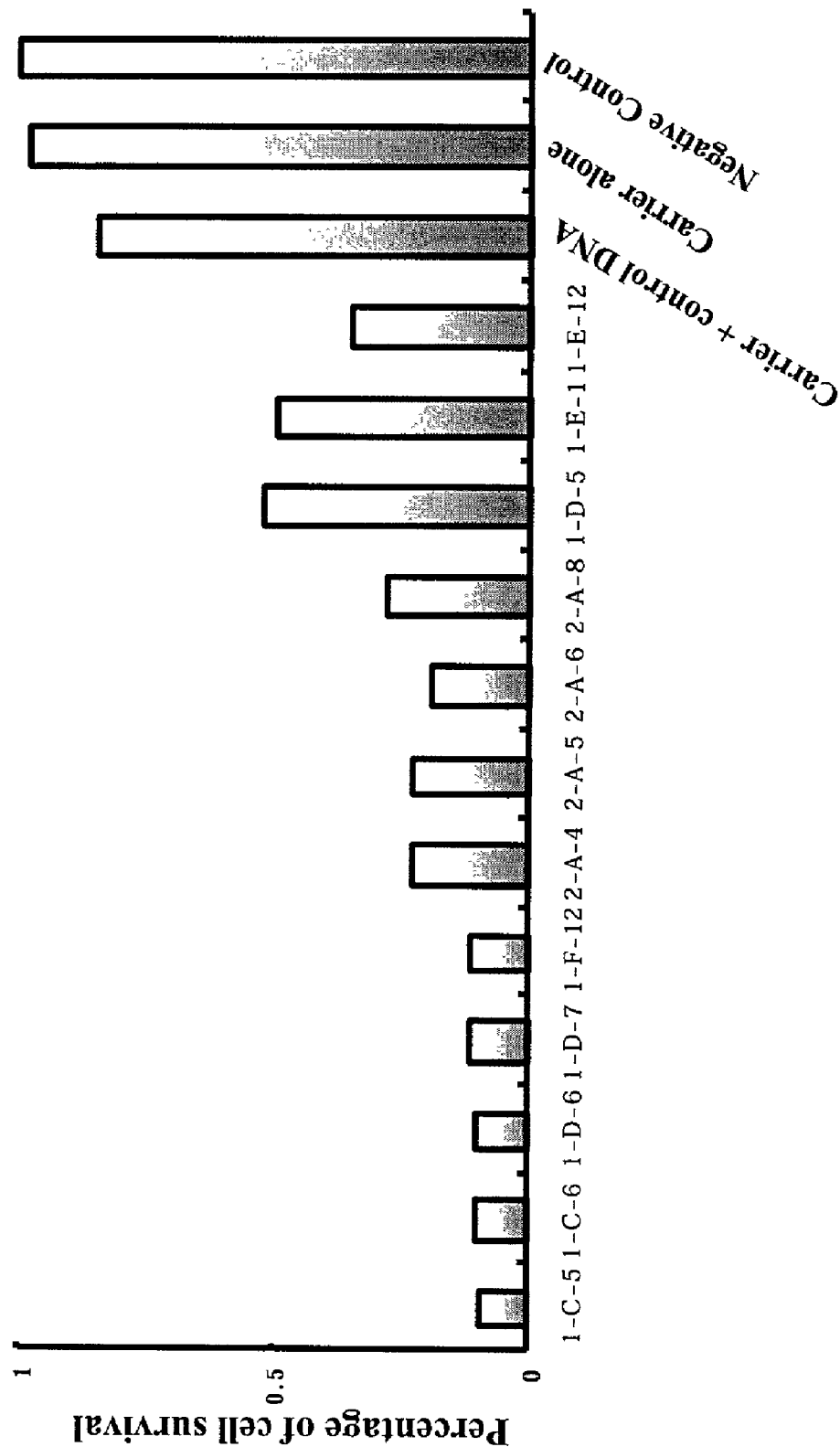


Fig. 17
(Random clone number 1)
HepG2 cell line

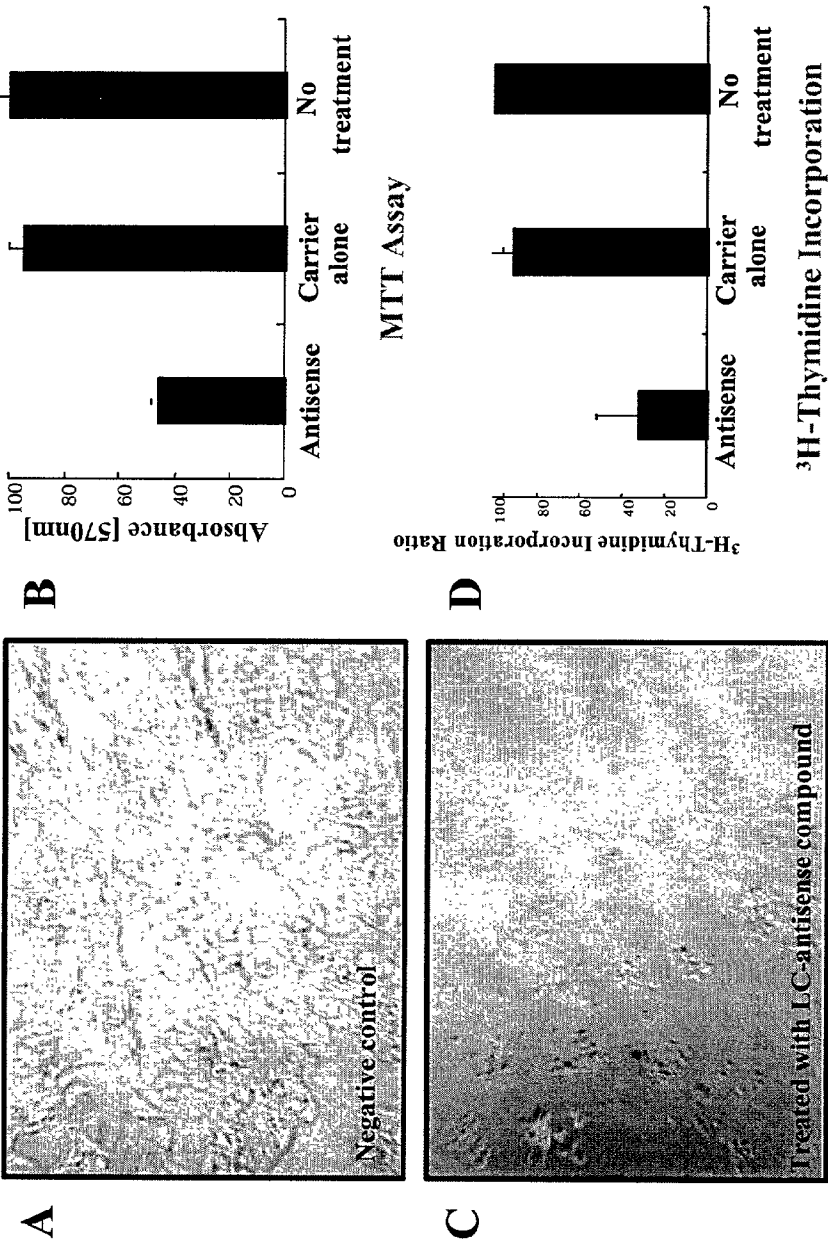


Fig. 18
(Random clone number 2)

HepG2 cell line

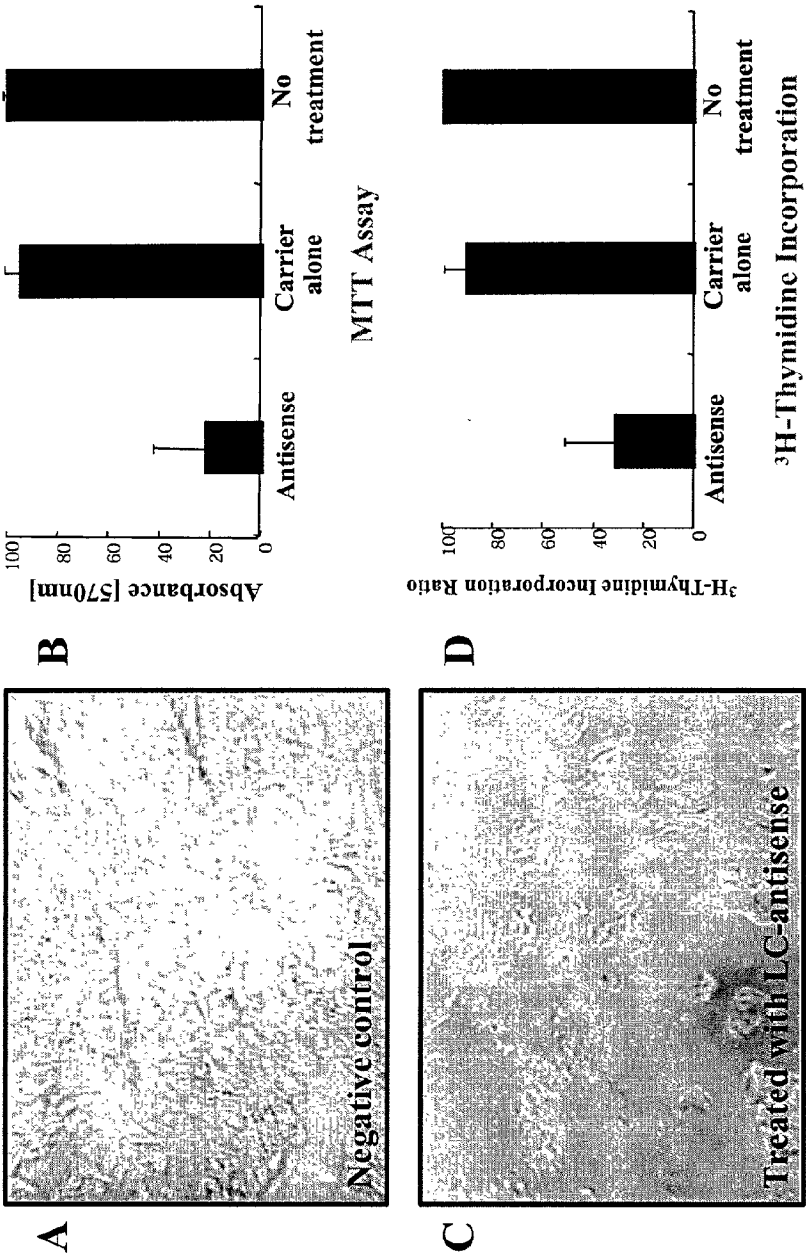
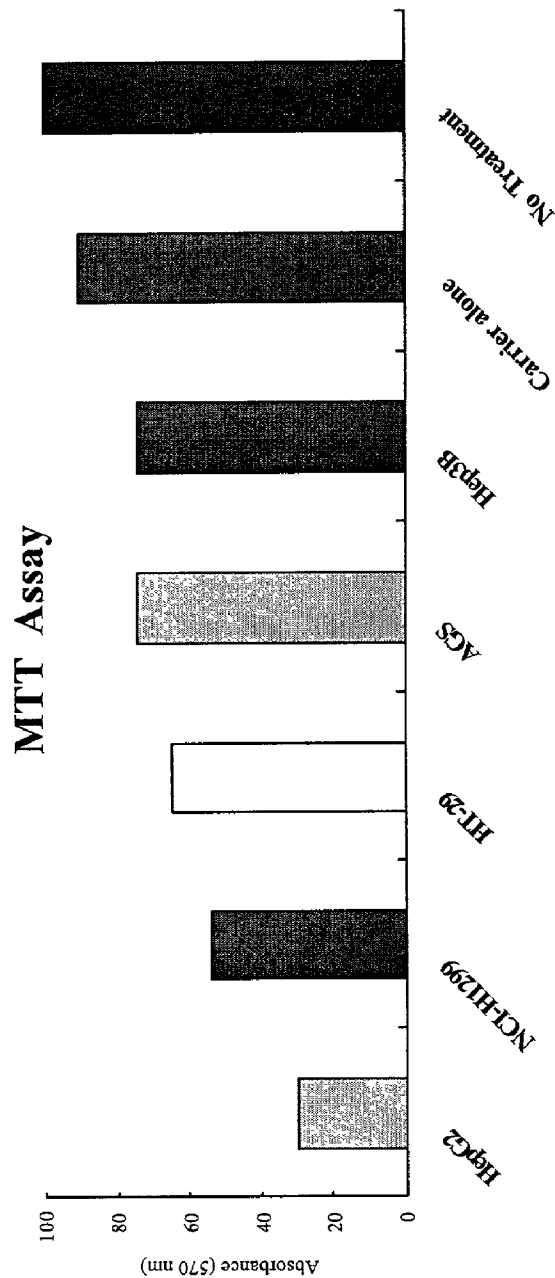


Fig. 19
Antisense activity profile of random clone number 3
against various types of cancer cells



RANDOM GENE UNIDIRECTIONAL ANTISENSE LIBRARY

BACKGROUND OF THE INVENTION

[0001] 1. Field of the Invention

[0002] The present invention relates to the field of antisense technology. The present invention also relates to using the antisense technology in therapeutics and in gene function identification systems. The present invention relates to a high-throughput system for functional genomics using a random gene unidirectional antisense library. And in particular, the present invention relates to a system for massively screening genes for their functions.

[0003] 2. Description of the Background

[0004] As most of the genetic information in human genome has been deciphered, many new methods for screening genes and analyzing their functions have been studied and developed at different institutions in the world. These methods have provided new information to understand the biochemical and physiological mechanisms of cell viability and the etiology of diseases. The molecular bases of many incurable diseases will be better understood and concomitantly more effective therapeutic agents will be developed.

[0005] Most human diseases are caused by abnormal gene expression. Genetic causes of disease are manifest by a variety of ways such as termination of gene expression by direct DNA damage, and abnormal transcription and/or translation. Abnormal expression of proto-oncogene expression can cause cancer (Brown et al., *Proc. Natl. Acad. Sci. USA*, 87(2), 538-542 (1990); Adams et al., *Proc. Natl. Acad. Sci. USA*, 80(7), 1982-1986 (1983)). It was reported that the occurrence and progression of immune-diseases are closely related to the overproduction or underproduction of cytokines (Pulsatelli et al., *J. Rheumatol.*, 26(9), 1992-2001 (1999)). Chronic or incurable diseases, such as various type of cancer, immune diseases, are caused by abnormal gene expression. Therefore, it is conceivable that these diseases can be controlled by modulation of gene expression.

[0006] It is known in the art that one way to control gene expression is by introducing to cells antisense oligos that are complementary to specific mRNA so that the antisense oligonucleotide may bind to the target mRNA and thus eliminate the target mRNA.

[0007] Antisense molecules bind to complementary sequences of mRNA through Watson-Crick base pairing. Antisense oligonucleotides (AS-oligos) have been valuable in the functional study of a gene by reducing gene expression in sequence specific manner (Thompson et al. *Nature*, 314, 363-366 (1985); Melani et al. *Cancer Res.*, 51, 2897-2901 (1991); Anfossi et al. *Proc. Natl. Acad. Sci. USA*, 86, 3379-3383 (1989)). Intense effort has also been made to develop antisense anticancer agents that eliminate aberrant expression of genes involved in tumor initiation and progression (Kamano et al. *Leuk. Res.*, 14, 831-839 (1990); Melotti et al. *Blood*, 87, 2221-2234 (1996); Ferrari et al. *Cell growth Differ.*, 1, 543-548 (1990); Ratajczak et al. *Blood*, 79, 1956-1961 (1992); Kastan et al. *Blood*, 74, 1517-1524 (1989); Thaler et al. *Proc. Natl. Acad. Sci. USA*, 93, 1352-1356 (1996); Wagner *Nature*, 372, 333-335 (1994)). Synthetic AS-oligos have been widely utilized for their ease of design and synthesis as well as potential specificity to a

target gene. Antisense inhibition of gene expression is believed to be achieved either through RNaseH activity following the formation of antisense DNA-mRNA duplex or through steric hindrance of the mRNA movement to bind a ribosomal complex (Dolnick *Cancer Invest.*, 9, 185-194 (1991)). There has also been an effort to inhibit gene expression by employing oligonucleotides that form triple helix aimed at the promoter region of the genomic DNA. Moreover, duplexed oligonucleotide decoys that compete with the promoter region of genomic DNA has also been formed (Young et al. *Proc. Natl. Acad. Sci. USA*, 88, 10023-10026 (1991)). Efficacy of AS-oligos has been validated in animal models as well as several recent clinical studies (Offensperger et al. *EMBO J.*, 12, 1257-1262 (1993); Tomita et al. *Hypertension*, 26, 131-136 (1995); Nesterova et al. *Nat. Med.*, 1, 528-533 (1995); Roush *Science*, 276, 1192-1193 (1997)). In addition, the first antisense drug was approved for CMV retinitis in US and Europe.

[0008] Expectations for AS-oligos have, however, frequently met with disappointment, as results have not always been unambiguous. Some of the problems of using AS-oligos have been inaccessibility to a target site (Flanagan et al. *Mol. Cell Biochem.*, 172, 213-225 (1997); Matsuda et al. *Mol. Biol. Cell*, 7, 1095-1106 (1996)), instability to nucleases (Akhtar et al. *Life Sci.*, 49, 1793-1801 (1991); Wagner et al. *Science*, 260, 1510-1513 (1993); Gryaznov et al. *Nucleic Acids Res.*, 24, 1508-1514 (1996)), lack of sequence specificity and various side effects in vivo. The stability of AS-oligos has improved to a certain extent by using chemically modified oligos, which are the so-called second generation AS-oligos (Helene *Eur J Cancer*, 27(11), 1466-71 (1991); Baker et al. *Biochim. Biophys. Acta.*, 1489, 3-18 (1999)). Phosphorothioate (PS)- and methylphosphonate (MP)-oligos, have been exhaustively studied and are utilized mainly to augment stability to nucleases. However, each of the modified AS-oligos exhibit both lack of sequence specificity and insensitivity to RNaseH. Further, there has been concern over inadvertent introduction of mutations during DNA replication or repair caused by recycling of hydrolyzed modified nucleotides.

[0009] A series of distinct antisense molecules with enhanced stability, the so-called 'third generation AS-oligos', having 1) a stem-loop structure, 2) the CMAS (Covalently-closed Multiple Antisense) structure and 3) the RiAS (Ribbon Antisense) structure (Moon et al. *Biochem J.*, 346, 295-303 (2000); Matsuda et al. *Mol. Biol. Cell*, 7, 1095-1106 (1996); Moon et al. *J Biol Chem.*, 275(7), 4647-53 (2000)) have been described. Both CMAS and RiAS-oligonucleotides exhibit enhanced stability to exonucleases and nucleases in biologic fluids. These antisense molecules are also efficacious in the specific reduction of target mRNA. However, there is a need in the art to develop an antisense molecule with greater facility and enhanced binding efficiency.

[0010] Certain bacteriophages, such as M13 bacteriophage, have a single-stranded circular genome, which has been employed for DNA sequencing analyses as well as mutagenesis studies. M13 phagemid, which is a plasmid used in the construction of a recombinant bacteriophage, can be engineered to produce a large quantity of circular single-stranded genomic DNA that contains an antisense sequence to a specific gene. This approach for producing antisense DNA takes advantage of the stability to exonucleases associated

with the covalently closed structure, high sequence fidelity, elimination of laborious target site search and easy construction of an antisense library.

[0011] Synthetic AS-oligos are about 15 to 25 bases long, and bind only to a single target site and eliminate substrate mRNA. However, most chronic and end stage human diseases show multiple genetic disorders. Thus, antisense molecules that can target multiple genes would appear to be more effective in treating such diseases. In order to satisfy such a need, it would be attractive to devise an antisense molecular system with multiple targeting ability. However, synthesizing such molecules would not be practical because of the difficulty chemically synthesizing them.

[0012] AS-oligos have a fundamental and inherent drawback for use in functional genomics. First, chemically modified AS-oligos cause nonspecific binding to irrelevant mRNA and as a result, they are less effective and often cytotoxic to cells, which of course creates false positive results. Second, synthetic AS-oligos, due to their short size (usually 15 to 25 bases), may not be uniformly effective in binding to their targets because they require a search before effectively binding to their target mRNA. Third, there is a possibility that an error in synthesis of AS-oligos decreases the specificity of their binding. Fourth, production cost for AS-oligo is high. And finally, when AS-oligos are used in functional genomics, these AS-oligos sometimes show incomplete antisense activity against their target mRNAs, thus generating unreliable and ambiguous data.

[0013] Current functional genomics systems using DNA chip technology, proteomics and so on are limited to providing gene expression profiles. However, to perform definitive functional analysis of genes, additional assays are required to be performed downstream of a particular gene inactivation.

[0014] Thus, there is a need in the art for a gene functionalization system to determine directly the functions of yet uncharacterized genes on a large scale.

SUMMARY OF THE INVENTION

[0015] The claimed invention overcomes the above-mentioned problems, and provides antisense molecules, compositions of antisense molecules, a method of making the antisense molecules, and a method of using the claimed molecules and compositions which provide the advantage of inhibiting or significantly modifying the expression of certain targeted genes. In the case that expression of these targeted gene(s) is responsible for causing cancer, then administering the inventive antisense molecules to the cells results in the ablation of the target RNA, which will inhibit proliferation of the cells, which in turn will result in curing or at least improving the survival associated with the cancer.

[0016] Applicants have developed large circular nucleic acid molecules that contain at least one target-specific antisense region by using a phagemid vector having a single-stranded circular genome. This large circular nucleic acid molecule may be called an LC-antisense compound. In a particular embodiment of the invention, applicants have constructed a phage genomic antisense library using cDNA from diseased tissue. The random gene antisense library was constructed unidirectionally. Further, in another embodiment of the invention, the cDNA pool was subtracted for

commonly expressed genes in control cells. The random gene unidirectional antisense library of the invention allows screening and analysis of the functions of genes with speed and accuracy, thus high-throughput and massive functional genomics systems are provided. Furthermore, the present invention may be used for validating therapeutic antisense compounds for chronic or incurable diseases.

[0017] Thus, in one aspect, the present invention is directed to a massive functional genomics method using LC-antisense compounds. LC-antisense compounds provide an effective platform for functionalization of a large number of genes with previously unknown functions and of genes with known and additional unknown functions. In addition, the present invention also may be used for the development of antisense molecular therapeutics and functional diagnostic systems.

[0018] LC-antisense compounds show superior antisense activity even with small doses as compared with conventional AS-oligos. Since typically, LC-antisense compounds are derived from cloned cDNAs in a phagemid vector, large single-stranded DNAs with target-specific antisense regions are generated. Thus, due to the large size of the molecule, LC-antisense compounds do not require a target site search for effective antisense activity. LC-antisense molecules are stable to exonucleases because they are covalently closed circular molecules. In addition, antisense libraries can be constructed relatively easily by introducing tens of thousands of different genes or gene fragments into phagemid vectors all at once. Large-scale generation of bacterially produced LC-antisense compounds can be easily obtained at low cost. Finally, the bacteriophage genomic antisense compound as applied to the area of massive functional genomics provides speed, low cost, and analytical accuracy.

[0019] It is to be understood that as the LC-antisense compounds are used therapeutically, the invention is not limited to treating cancer. The principles of the antisense compound of the invention may be applied to efficiently ablate any target RNA. Any phenotypic manifestation of this chemical activity in the form of cancer treatment, eliminating adverse effects of viral infection, treating metabolic diseases, immunologic disorders, and so on may be the result of antisense molecular therapy.

[0020] The LC-antisense compounds chosen from a large antisense library may be adapted to configure an antisense macroarray system. The antisense macroarray system may be effectively utilized for functional comparison of the antisense compounds among different types of cells treated with the antisense compounds. Comparative functional diagnostics as well as understanding the underlying molecular mechanism of a disease may be performed by employing the antisense macroarray assembly system of the invention.

[0021] A panel of antisense compounds used in the antisense macroarray assembly may be chosen based on the results obtained from either a primary functional assay using an antisense library or from conventional expression profiling or expression tracking system, such as DNA chip, SAGE, Toga and proteomics.

[0022] The invention further includes compositions of the claimed antisense molecules together with a pharmaceutically acceptable carrier. In one aspect of the invention the invention is directed to a library of a multitude of single-stranded large circular nucleic acids, said library comprising:

[0023] a multiplicity of compartments, each of said compartments comprising one or more single-stranded large circular antisense molecule of bacteriophage or phagemid vector comprising at least one unidirectional antisense nucleic acid insert,

[0024] wherein said large circular antisense molecule is capable of being introduced into a host cell, and is capable of specifically binding to a nucleic acid in said host cell that is substantially complementary to said antisense nucleic acid insert.

[0025] In the library discussed above, the specificity of the antisense nucleic acid insert may be unknown at the time said library is first made. Further, the host cell may be a eucaryotic cell. And each compartment may contain from about 0.1 μ M to about 1 μ M of the large circular antisense molecule, preferably in an aqueous medium. The bacteriophage or phagemid vector may be derived from a filamentous bacteriophage. And the filamentous bacteriophage may be an M13 bacteriophage. Furthermore, the bacteriophage or phagemid vector may comprise more than one kind of antisense nucleic acid insert sequence. In addition, in the library discussed above, the source of the nucleic acid insert may be an eucaryotic organism.

[0026] The library may also contain multiple compartments, wherein the compartments may be a multiwell format of, without limitation, 6 wells, or preferably, 96 wells. The library may be also configured to be made and used in a substantially automated process.

[0027] In another embodiment, the invention is directed to a method of making a library comprising a multitude of single-stranded large circular nucleic acids, which comprises one or more single-stranded bacteriophage or phagemid vector comprising at least one unidirectional antisense nucleic acid insert, comprising:

[0028] (i) inserting a nucleic acid fragment unidirectionally into said bacteriophage or phagemid vector by unidirectionally cloning the nucleic fragments into said vector;

[0029] (ii) preparing bacterial transformants by introducing the vector containing the insert into competent bacterial cells to make bacterial transformants; and

[0030] (iii) infecting said transformants with helper phage to produce said single-stranded nucleic acid library.

[0031] In yet another embodiment, the invention is directed to a library of a multitude of single-stranded large circular nucleic acids, said library comprising:

[0032] a multiplicity of compartments, each of said compartments comprising one or more single-stranded large circular antisense molecule of bacteriophage or phagemid vector comprising at least one unidirectional subtracted antisense nucleic acid insert,

[0033] wherein said large circular antisense molecule is capable of being introduced into a host cell, and is capable of specifically binding to a nucleic acid in said host cell that is substantially complementary to said antisense nucleic acid insert.

[0034] In the library above, the unidirectional subtracted antisense nucleic acid may be made by hybridizing a population of nucleic acids expressed from a first cell line or tissue with a population of nucleic acids expressed from a second cell line or tissue, and obtaining a nucleic acid population from the first cell line or tissue that does not hybridize with the nucleic acid population from said second cell line or tissue.

[0035] In particular, the first cell line or tissue may be abnormal such that modulation of gene expression is beneficial in returning said first cell line or tissue to normal, and wherein said second cell line or tissue is normal. The abnormality may be cancer, viral infection, immunologic disorders or metabolic diseases. And cancer may be liver cancer, lung cancer, stomach cancer, colon cancer, leukemia, thyroid cancer, skin cancer, prostate cancer, cervical cancer, or breast cancer. Viral infection may be caused by human papilloma virus (HPV), HIV, small pox, mononucleosis (Epstein-Barr virus), hepatitis, or respiratory syncytial virus (RSV). And metabolic disease may be phenylketonuria (PKU), primary hypothyroidism, galactosemia, abnormal hemoglobins, types I and II diabetes, or obesity. Also in particular, the immunological disorder may be Sjogren's Syndrome, antiphospholipid syndrome, immune complex diseases, Purpura, Schoenlein-Henoch, immunologic deficiency syndromes, systemic lupus erythematosus, immunodeficiency, rheumatism, kidney, or liver sclerosis.

[0036] In still another embodiment, the invention is directed to a method of making a library comprising a multitude of single-stranded large circular nucleic acids, which comprises one or more single-stranded bacteriophage or phagemid vector comprising at least one unidirectional subtracted antisense nucleic acid insert, comprising:

[0037] (i) inserting a subtracted nucleic acid fragment unidirectionally into said bacteriophage or phagemid vector by unidirectionally cloning the subtracted nucleic fragments into said vector;

[0038] (ii) preparing bacterial transformants by introducing the vector containing the insert into competent bacterial cells to make bacterial transformants; and

[0039] (iii) infecting said transformants with helper phage to produce said single-stranded nucleic acid library.

[0040] In the method above, the subtracted nucleic fragment may be made by hybridizing a population of nucleic acids expressed from a first cell line or tissue with a population of nucleic acids expressed from a second cell line or tissue, and obtaining a nucleic acid population from the first cell line or tissue that does not hybridize with the nucleic acid population from said second cell line or tissue.

[0041] In a further aspect, the invention is directed to a method for specifically inhibiting growth of liver cancer cells, comprising administering to said cells large circular antisense molecules targeted to EST_Human IL3-UTO117-160301-504-H11; Apolipoprotein A-II, clone MGC:12334; PRO2675 mRNA; clone RP11-449G13 from 16; BAC clone RP11-360H4 from 2; gene supported by AK023036 (LOC90271); or gene similar to cytochrome b5 outer mitochondrial membrane precursor (H. sapiens) (LOC124229).

[0042] In yet a further aspect, the invention is directed to a method for specifically inhibiting growth of liver cancer cells, comprising administering to said cells large circular antisense molecules targeted to HSPC025, clone MGC:4223 IMAGE:2959747; tissue inhibitor of metalloproteinase 1; alpha-fetoprotein (AFP); gene encoding protein FLJ14075; apolipoprotein A-II (APOA2); clone MGC:20176 IMAGE:3503710; eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2); cytochrome P450, subfamily IIE (ethanol-inducible) (CYP2E); or gene similar to serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1, clone MGC:9222 IMAGE:3859644.

[0043] The invention is also directed to a high throughput system for functional genomics using a random gene unidirectional antisense library or random gene unidirectional subtracted antisense library comprising the following steps:

[0044] (i) forming large circular antisense molecule-carrier complexes with said unidirectional or unidirectional subtracted antisense libraries;

[0045] (ii) performing a primary gene functional analysis by transfecting the complexes into host cells to screen for the large circular antisense molecule that eliminates endogenously expressed substantially complementary transcripts;

[0046] (iii) identifying the large circular antisense molecule that eliminates the endogenously expressed transcript; and

[0047] (iv) sequencing either the antisense molecule or cDNA that corresponds to the antisense molecule.

[0048] In the high throughput system described above, the system may further comprise step (v) of performing further gene function analysis with the large circular antisense molecule identified in steps (iii) and (iv). Still further, comprising comparing the gene sequence obtained in step (iv) with a DNA sequence database to identify the gene.

[0049] The carrier may be, without limitation, liposomes, cationic polymers, HVJ-liposomes complexes, peptides or viruses. Further, in a specific embodiment, the large circular antisense molecule and carrier may be mixed in an optimal ratio of about 1:3 to about 1:4 by weight.

[0050] In a specific embodiment of the invention, the gene function analysis may be assaying for the phenotype of cell morphology, cell proliferation, cell apoptosis, or cell reaction to a substrate. And in particular, the gene function analysis may be carried out by performing an assay, wherein said assay is RT-PCR, Western blot analysis, immunoassay, MTT reduction assay, [³H]-thymidine incorporation assay, colony formation assay, DNA synthesis and chromatin activation, analysis of apoptosis by inspection of cell morphological changes, chromosomal condensation or fragmentation, DNA fragmentation, quantitative assay for apoptosis, signaling mechanisms of apoptosis, activation of cell cycle regulators, complex formation between cell cycle regulators, or assays for changes of metabolic, morphological, physiological and biochemical phenotypes in vitro and in vivo.

[0051] These and other objects of the invention will be more fully understood from the following description of the invention, the referenced drawings attached hereto and the claims appended hereto.

BRIEF DESCRIPTION OF THE DRAWINGS

[0052] The present invention will become more fully understood from the detailed description given herein below, and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein;

[0053] FIG. 1 shows a schematic diagram for generating rat TNF α -M13 antisense molecule (TNF α -M13AS).

[0054] FIG. 2 shows sequence analysis of TNF α -M13AS confirming the antisense nature of the TNF- α insert.

[0055] FIG. 3A shows the results of treating TNF α -M13AS with various enzymes. TNF α -M13AS was confirmed to be single-stranded. Lane 1: Plasmid DNA containing TNF α -cDNA (TNF α -plasmid); Lane 2: TNF α -M13AS; Lane 3: TNF α -plasmid digested with Xho I; Lane 4: TNF α -M13AS digested with Xho I; Lane 5: TNF α -plasmid digested with S1 nuclease; Lane 6: TNF α -M13AS digested with S1 nuclease; Lane 7: TNF α -plasmid digested with Xho I and exonuclease III; and Lane 8: TNF α -M13AS digested with Xho I and exonuclease III.

[0056] FIG. 3B shows the stability of TNF α -M13AS to nucleases. Lane 1: TNF α -plasmid; Lane 2: TNF α -M13AS; Lane 3: TNF α -plasmid+FBS; Lane 4: TNF α -plasmid digested with Xho I+FBS; Lane 5: TNF α -M13AS+FBS; Lane 6: TNF α -M13AS and liposome complex+FBS; Lane 7: TNF α -plasmid+calf serum; Lane 8: TNF α -plasmid digested with Xho I+calf serum; Lane 9: TNF α -M13AS+calf serum; and Lane 10: TNF α -M13AS and liposome complex+calf serum.

[0057] FIG. 4A shows the results of RT-PCR using a TNF α -specific primer pair and a β -actin specific primer pair. Rat TNF- α expression was specifically inhibited by TNF α -M13AS of the present invention. Lane 1: Liposome; Lane 2: TNF α -M13AS; Lane 3: TNF α -M13 sense; and Lane 4: Single-stranded phage genomic DNA without rat TNF- α cDNA.

[0058] FIG. 4B shows the results of amplifying IL-1 β and GAPDH transcripts by RT-PCR, confirming that TNF α -M13AS specifically inhibits the expression of rat TNF- α . Lane 1: Liposome; Lane 2: TNF α -M13AS; Lane 3: TNF α -M13 sense; and Lane 4: Single-stranded phage genomic DNA without rat TNF- α sequence.

[0059] FIG. 4C shows Southern blot data using rat TNF- α specific hybridization probe, confirming that TNF α -M13AS specifically inhibits the expression of rat TNF- α . Lane 1: Liposome; Lane 2: TNF α -M13AS; Lane 3: TNF α -M13 sense; and Lane 4: Single-stranded phage genomic DNA without rat TNF- α cDNA.

[0060] FIG. 5 shows ELISA assay data that measure the quantity of rat TNF- α protein secreted from cells. The data show that rat TNF- α protein expression decreases in response to administration of TNF α -M13AS.

[0061] FIG. 6A shows RT-PCR results confirming that endogenous NF- κ B expression decreases in response to administration of NF κ B-M13AS.

[0062] FIG. 6B shows confirmatory Southern blot results using an NF- κ B specific probe. Data confirm that human NF- κ B expression decreases in response to administration of NF κ B-M13AS.

[0063] FIG. 7 shows a schematic diagram of a high-throughput system for functional genomics using a random gene unidirectional antisense library.

[0064] FIG. 8 shows a schematic diagram of gene functionalization method using random gene unidirectional antisense library.

[0065] FIG. 9 shows a strategy for constructing a random gene unidirectional antisense library.

[0066] FIG. 10 shows a strategy for constructing a random gene unidirectional subtracted antisense library.

[0067] FIG. 11 shows the quality of the unidirectional subtracted liver cDNA library. To determine the percentage of vectors with cDNA inserts, 40 randomly selected recombinant phagemid clones were purified and digested with Not I and Xho I, and electrophoresed on a 1% agarose gel.

[0068] FIG. 12 shows sample sequence analyses of the unidirectional subtracted liver cDNA library. The cDNA regions in pBluescript (pBS) SK(−) were sequenced from the 5' end of the (+) strand by employing T3 primer. Each cDNA sequence was compared with Genbank database.

[0069] FIG. 12A shows human RBP 56/hTAF II (human RBP 56/hTAF II) gene insert.

[0070] FIG. 12B shows the α -fetoprotein gene insert.

[0071] FIG. 12C shows *Homo sapiens* chromosome 17, clone hC gene insert.

[0072] FIG. 13 shows selection of cDNA clones having insert sizes above 500 bases by 'cracking' method, which is a type of multiple mini-scale plasmid preparation method. Recombinant phagemid with 500 bp of cDNA was used as a control.

[0073] FIG. 14 shows a random gene unidirectional subtracted liver antisense library comprising LC-antisense compounds derived from clones selected by the 'cracking' method.

[0074] FIGS. 15A-15I show inhibition of cell proliferation of liver cancer cells (HepG2) after transfection with LC-antisense compound-carrier complexes. This is an example of primary functional analysis of genes. Changes in cell proliferation were observed by visual observation using light microscopy (original magnification, X200) after 4th day of transfection. FIGS. 15A-15C show negative control cells. FIGS. 15D-15I show HepG2 cells treated with LC-antisense compounds.

[0075] FIG. 16 shows massive gene functionalization using random gene unidirectional subtracted liver antisense library. The liver cancer cell line (HepG2) was transfected with either an LC-antisense compound-carrier complex or control carrier compounds in a 96-well plate. MTT assay was carried out to observe changes in cell proliferation. Genes related to liver cancer cell growth were identified by calculating the percentage of growth inhibition.

[0076] FIGS. 17A-17D show massive gene functionalization using a random clone number 1 from random gene unidirectional subtracted liver antisense library. Measurement of cell growth inhibition of HepG2 was performed by observation with light microscopy (FIGS. 17A and 17C), MTT assay (FIG. 17B) and [H]-thymidine incorporation assay (FIG. 17D).

[0077] FIGS. 18A-18D show gene functionalization using a random clone number 2 from random gene unidirectional subtracted liver antisense library. Measurement of cell growth inhibition of HepG2 was performed by observation with light microscopy (FIGS. 18A and 18C), MTT assay (FIG. 18B) and [³H]-thymidine incorporation assay (FIG. 18D).

[0078] FIG. 19 shows antisense activity profile of an example of LC-antisense compound designated clone number 3 to various kinds of cancer cells. A macroarray comprising LC-antisense compounds to 80 identified gene clones involved in the cell growth of liver cancer was transfected via a pharmaceutically accepted carrier into different cancer cell lines, Hep3B (liver cancer), NCI-H1299 (non-small lung cancer), AGS (stomach cancer) and HT-29 (colon cancer). Cell growth was measured using MTT assay on a macroarray assembly, and data were compared to study the antisense activity profile of these LC-antisense compounds.

DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0079] The present invention is based on the discovery that a large circular phage genomic molecule that includes a target specific antisense region, is useful as an effective ablator of gene expression, and as such can be used to determine the function of a target gene. The inventive system can be used in a high-throughput manner in a massive functional genomics protocol to determine genes involved in various cellular physiological processes.

[0080] In particular, the present invention provides LC-antisense compounds derived from recombinant bacteriophage genome and methods for preparing them. The present invention also provides at least two kinds of antisense libraries. One is unidirectional antisense library and another is unidirectional subtracted antisense library, both of which may be constructed using bacteriophage genome antisense vectors. Additionally, the present invention provides a high-throughput system for functional genomics using the antisense libraries.

[0081] In the present application, "a" and "an" are used to refer to both single and a plurality of objects.

[0082] As used herein, the term "antisense" means antisense nucleic acid (DNA or RNA) and analogs thereof and refers to a range of chemical species having a range of nucleotide base sequences that recognize polynucleotide target sequences or sequence portions through hydrogen bonding interactions with the nucleotide bases of the target sequences. The target sequences may be single- or double-stranded RNA, or single- or double-stranded DNA.

[0083] Such RNA or DNA analogs comprise but are not limited to 2'-O-alkyl sugar modifications, methylphosphonate, phosphorothioate, phosphorodithioate, formacetal, 3'-thioformacetal, sulfone, sulfamate, and nitroxide backbone modifications, amides, and analogs wherein the base moieties have been modified. In addition, analogs of molecules may be polymers in which the sugar moiety has been modified or replaced by another suitable moiety, resulting in polymers which include, but are not limited to, morpholino analogs and peptide nucleic acid (PNA) analogs. Such analogs include various combinations of the above-men-

tioned modifications involving linkage groups and/or structural modifications of the sugar or base for the purpose of improving RNaseH-mediated destruction of the targeted RNA, binding affinity, nuclease resistance, and or target specificity.

[0084] As used herein, “antisense therapy” is a generic term, which includes specific binding of large circular antisense molecules that include an antisense segment for a target gene to inactivate undesirable target DNA or RNA sequences in vitro or in vivo.

[0085] As used herein, “cell proliferation” refers to cell division. The term “growth,” as used herein, encompasses both increased cell numbers due to faster cell division and due to slower rates of apoptosis, i.e. cell death. Uncontrolled cell proliferation is a marker for a cancerous or abnormal cell type. Normal, non-cancerous cells divide regularly, at a frequency characteristic for the particular type of cell. When a cell has been transformed into a cancerous state, the cell divides and proliferates uncontrollably. Inhibition of proliferation or growth modulates the uncontrolled division of the cell.

[0086] As used herein, “chimeric large circular antisense molecule” refers to a large circular nucleic acid molecule comprising a plurality of antisense nucleotide segments that are substantially complementary to a plurality of target genes. The segments of antisense nucleotides may be connected or linked to each other directly or indirectly by use of spacers between each segment.

[0087] As used herein, “compartment” or “compartments” refers to a physical delineation of each member clone of the LC-antisense molecular library. Physical delineation may be in the form of wells such as in multiwell plates. Commonly used are 96 well plates or 96 deep well plates. Another physical barrier may be air, such as by individual spotting on a flat sheet or membrane. In this regard, either macroarray or microarray methods may be used. It is understood that by compartmentalization it is meant that the clone members are separated from each other. Other barriers may be by encapsulation of individual clones in a membranous material, and the like.

[0088] As used herein, “filamentous phage” is a vehicle for producing the large circular antisense molecule of the invention. Phages or phagemids may be used. In this instance, the desired sequence is inserted or cloned into the vehicle so that when a single strand is generated by the phage or phagemid, the large circular antisense molecule is generated. DNA or RNA bacteriophage may be used for this purpose. In particular, filamentous bacteriophage may be used. Filamentous phages such as M13, fd, and f1 have a filamentous capsid with a circular ssDNA molecule. Their life-cycle involves a dsDNA intermediate replicative form within the cell which is converted to a ssDNA molecule prior to encapsidation. This conversion provides a means to prepare ssDNA. The bacteriophage M13 has been adapted for use as a cloning vector.

[0089] Phagemid vectors also have filamentous phage fl Ori region. pBluescript (Stratagene, USA), pGEM-f (Promega, USA), M13mp, pCR2.1, pGL2, pβgal and pSPORT vector and their derivatives may be used. Preferentially, the phagemid vector of M13 bacteriophage, pBluescript SK(-), may be used. One advantage of using a

recombinant viral vector based on M13 bacteriophage is that the vector can accommodate a variety of sizes of antisense inserts. Because pBluescript SK(-) phagemid vector has fl(-) origin, the entire nucleotide sequence comprising the antisense form of the target nucleotide sequence and vector originated genes, for example, the ampicillin resistance gene and the lacZ gene, are expressed in a single-stranded form.

[0090] Another bacteriophage having single-stranded circular genome and having an icosahedral shape is ΦDX174. However, this cloning vector has a limitation on the insert size.

[0091] As used herein, “functional genomics” or “massive functional genomics” refers to the scientific discipline and utility in biotechnology in which the functions of genes are experimentally determined and identified. If this process is performed with rapidity, in parallel, and in great quantities, it may be termed “high-throughput” or “massive” functional genomics.

[0092] As used herein, the terms “inhibiting” and “reducing” are used interchangeably to indicate lowering of gene expression or cell proliferation or any other phenotypic characteristic.

[0093] As used herein, “large circular antisense molecule (LC-antisense molecule)” also referred to as “phage genomic antisense molecule”, or sometimes “large circular nucleic acid molecule”, is a single-stranded molecule, which includes at least one antisense region that is substantially complementary to and binds a target gene or RNA sequence, which inhibits or reduces expression of the gene as well as, in some instances, its isoforms. The circular single-stranded nucleic acid molecule may contain either sense or antisense sequence for one or several genes, so long as the sequence for the target gene is in the antisense form.

[0094] Large circular nucleic acid molecule may be synthesized by chemical methods. Typically, however, it is produced from a filamentous phage system, which includes M13 and phagemids that are derived from it. When the large circular nucleic acid molecule is generated from a phage, it may also be referred to as a “phage genomic antisense compound”.

[0095] In one sense, the large circular nucleic acid molecule is longer than a typical oligonucleotide sequence that may be about 20 to 30 nucleotides long. In contrast, the large circular nucleic acid molecule may be at least 3,000 nucleotides long. Typically, the range may be from about 3,000 to about 8,000 nucleotides long. Although a length of about 3,100 to about 7,000 nucleotides may be useful in the invention, preferred length range may be from about 3,300 to about 6,000 bases.

[0096] Alternatively, it is understood that there does not have to be an absolute upper or lower limit to the length of the large circular nucleic acid molecule. This is especially so when a phage is used to generate the large circular nucleic acid molecule, in which case the size of the phage and the size of the insert that encodes at least a portion of the target gene may control the length of the single-stranded nucleic acid generated. Thus, in one embodiment, the nucleic acid molecule may be as long as a phage such as a filamentous phage may accommodate.

[0097] The large circular nucleic acid molecule may contain both the specific antisense sequence as well as extra-

neous sequence. Extraneous sequence may include sense or antisense forms of various other genes. Or, if a phage is used to generate the nucleic acid molecule, the extraneous sequence may be the vector sequence. The length of the target specific antisense region of the large circular nucleic acid molecule may be without limitation from a bit lower than about 100 nucleotides to over about 5,000 bases. Typically, the range may be from about 200 to about 3,000. In particular, the range may be about 400 to about 2,000. The target specific antisense region may be also complementary to an entire gene.

[0098] In another embodiment, the antisense molecule may be generated from the genome of a bacteriophage as part of the natural life cycle of the phage.

[0099] As used herein, "macroarray" refers to a selected set of LC-antisense compounds, which can be employed to examine functional profile of the antisense molecules in different types of cells or cell lines.

[0100] As used herein, a "gene" refers either to the complete nucleotide sequence of the gene, or to a sequence portion of the gene.

[0101] As used herein, "substantially complementary" means an antisense sequence having about 80% homology with an antisense compound which itself is complementary to and specifically binds to the target RNA. As a general matter, absolute complementarity may not be required. Any antisense molecule having sufficient complementarity to form a stable duplex or triplex with the target nucleic acid is considered to be suitable. Since stable duplex formation depends on the sequence and length of the hybridizing antisense molecule and the degree of complementarity between the antisense molecule and the target sequence, the system can tolerate less fidelity in complementarity with large circular antisense molecule.

[0102] As used herein, "unidirectional subtracted library" refers to a library that is selectively enriched for genes that are expressed or overexpressed in a particular tissue or cell line of interest as compared with a control tissue or cell line.

[0103] As used herein, "target" or "targeting" refers to a particular individual gene for which an antisense molecule is made. In an embodiment of the invention, the antisense molecule is made from an insert in a LC-antisense compound. In certain contexts, "targeting" means binding or causing to be bound the antisense molecule to the endogenously expressed transcript so that target gene expression is eliminated. The target nucleotide sequence may be selected from genes involved in various malignancies, including genes involved in the initiation and progression of various diseases such as immune diseases, infectious diseases, metabolic diseases and hereditary diseases or any other disease caused by abnormal expression of genes.

[0104] As used herein, "unidirectional" or "random gene unidirectional" antisense library refers to the uniformity of orientation of the insert genes in each member clone in the gene library. By the term "random", it is meant to refer to a library that contains genes of unverified sequence.

[0105] Large Circular (LC) Antisense Compounds

[0106] The present invention provides LC-antisense compounds having enhanced stability to nucleases and specific activity, and a method for producing the LC-antisense com-

pounds by using recombinant bacteriophages with single-stranded circular genome. Further, in one embodiment of the invention, by employing the phage genomic antisense method of the invention, the efficiency of the system as used in massive functional genomics is superior by several hundred fold to that of conventional AS-oligos method. Moreover, contrary to using other indirect systems, such as DNA chip, Serial Analysis of Gene Expression (SAGE), and TIGR Orthologous Gene Alignment (TOGA) database proteomics, massive functional genomics employing the inventive phage genomic antisense system employs a direct gene functionalization system.

[0107] The LC-antisense compounds of the present invention may be made by 1) preparing a cDNA fragment having a target nucleotide sequence; 2) preparing a recombinant phage by cloning the cDNA fragment in a phagemid vector that is capable of producing a LC-antisense compound; and 3) producing the single-stranded circular phage genome containing the target antisense sequence in a large scale manner.

[0108] It is understood that the LC-antisense compounds may comprise either fragments of a target sequence or the entire gene sequence. Also, it is contemplated that several target antisense sequences for a plurality of different genes may be inserted into one single-stranded phage genome.

[0109] LC-antisense compounds have strong replication fidelity because the compound is replicated by DNA polymerase in bacterial cells. Since DNA polymerase has proof reading capabilities, the fidelity of LC-antisense compound is greater than chemically synthesized AS-oligos. Moreover, LC-antisense compounds of the present invention are cheaper to make than the chemically synthesized oligonucleotides. High cost required for the synthesis of high quality AS-oligos has been regarded as an obstacle for preclinical and clinical trials.

[0110] LC-antisense compounds are stable against nucleases, and are target specific. In contrast, when chemically modified oligonucleotides are introduced to the cells, mutations as well as retardation of blood clotting or complement activation reaction are induced. Additionally, when the chemically synthesized oligonucleotides are eventually degraded, the individual nucleotides are recycled back into the genomic DNA through DNA replication or repair mechanisms. Incorporation of the chemically modified nucleotides into genomic DNA will likely cause mutations.

[0111] Without being bound by any particular theory regarding why the LC-antisense compounds have these advantageous properties, it is believed that when a large target-specific antisense sequence such as the LC-antisense compound of the invention is used, searching for an open site along the target mRNA is likely to be easily achieved.

[0112] In exemplified embodiments of the invention, LC-antisense compounds against TNF- α and NF- κ B were prepared. Each of these LC-antisense compounds was about 3.7 kb in size and was stable to nuclease degradation. The TNF- α specific insert was 708 bp, and was effective in ablating TNF- α gene expression. The NF- κ B specific insert was 700 bp, and it too was effective in ablating NF- κ B gene expression. This presents a significant advantage over using chemically synthesized oligonucleotides, which require a careful and laborious process of determining the effective

target sites. Thus, the LC-antisense compound is facile to use and saves time and effort associated with searching for effective target sites.

[0113] In addition, the efficiency of the liposome mediated delivery of LC-antisense compounds is close to that of a plasmid because of its sufficiently long sequence, which contributes to the excellent antisense activity associated with LC-antisense compounds. The rate of cellular uptake of LC-antisense compound-liposome complex was better than the rate of uptake of oligonucleotides.

[0114] LC-antisense compounds generally include the target antisense sequence and either antisense or sense form of the nucleotide sequences of the vector encoded genes such as ampicillin resistance gene and β -galactosidase gene (lacZ). However, LC-antisense compounds did not cause any significant amount of non-specific inhibition of gene expression. In contrast, chemically modified synthetic oligonucleotides cause significant problems associated with non-specific inhibition.

[0115] Regarding the size of the antisense molecule, conventional wisdom in the field of antisense research has discouraged using long antisense molecules because it was thought that longer AS-oligos tend to be less specific, harder to synthesize and inefficient in cellular uptake. Indeed, chemically modified second generation AS-oligos such as phosphorothioate modified oligos lose sequence specificity as the length of the AS-oligos is extended. Furthermore, synthesis of linear AS-oligos becomes increasingly difficult as the oligonucleotides are extended to longer sequences, and sequence fidelity declines markedly as the length of the AS-oligos increases. However, in contravention of this teaching, applicants have discovered that antisense activity is dependent on the length of the antisense sequence. If the length of the antisense sequence is decreased, the antisense activity also decreases. Thus, LC-antisense compounds exhibit sequence specificity, resistance to nuclease degradation, and non-toxicity.

[0116] Some of the significantly advantageous features of LC-antisense compounds are as follows:

[0117] 1. LC-antisense compounds have an improved antisense activity. Typically, without being limited by any specified amount, which amounts are offered herein as merely being exemplary of the practice of the invention, administration of approximately 1×10^5 cells with 0.1 μg of the antisense compound can achieve complete ablation of the target transcript. In addition, the antisense sequence may be less than one fifth the size of the entire length of the transcript. LC-antisense molecule has high antisense activity with respect to the amount of antisense compound that is administered.

[0118] 2. LC-antisense compounds can be produced massively with speed, accuracy and cost effectiveness from a bacterial transformant, such as *E. coli*.

[0119] 3. The LC-antisense compound-carrier complex is easily absorbed by cells.

[0120] 4. LC-antisense compounds are stable against nucleases in serum and can form stable complexes with liposomes.

[0121] 5. LC-antisense compounds are replicated by DNA polymerase in bacterial cells such as *E. coli*.

[0122] 6. Ablation of multiple target mRNA is achievable. A chimeric LC-antisense compound may contain a plurality of target-specific antisense sequences in a single vector. The length of each of the antisense sequences may be typically much longer than those of chemically modified antisense oligonucleotides. Several distinct antisense sequences can be located in series. Therefore, it is possible to target multiple types of transcripts of several different genes. This property can be of use in eliminating expression of multiple genes in incurable diseases such as advanced types of cancer exhibiting aberrant gene expression of multiple genes.

[0123] 7. LC-antisense compounds show low toxicity. Since LC-antisense compounds are composed of the same base composition found in nature, non-specificity and undesired toxic effects are reduced when compared with chemically modified AS-oligos.

[0124] 8. A random gene or unigene unidirectional antisense library can be easily constructed. Construction of an antisense library with a large number of individual clones may be performed easily and rapidly. A random gene unidirectional antisense library specific to a particular disease can be easily constructed from diseased cells or abnormal cells or tissue. Thus, the random gene antisense library may comprise antisense molecules to disease-specific genes. The member antisense compounds initially are not individually verified for their DNA sequences, and thus, some clones may be redundant. Meanwhile, a unigene antisense library of the entire panel of human genes or genes of other organisms may be rather extensive and constructed without redundancy among its member antisense compounds. These antisense libraries may include thousands or tens of thousands of cloned genes that may be employed for efficiently performing massive gene functionalization by knock-down of gene expression in particular cell types. In contrast, a significant drawback of using synthetic AS-oligos is the time-consuming requirement for the selection of a target site. Another drawback to using conventionally known AS-oligos is that its data can be misinterpreted because partial antisense activity sometimes occurs.

[0125] Random Gene Unidirectional Antisense Library

[0126] The present invention provides methods for the construction of a unidirectional antisense library and a random gene unidirectional subtracted antisense library using the phage genome. For constructing the antisense libraries, both randomly selected genes from a cDNA library and sequence-verified 'unigenes' are available as cDNA source.

[0127] Without being limited to using any particular phage system, in one embodiment, LC-antisense compounds are produced massively from a bacterial culture containing recombinant bacteriophages. For this purpose, the present

inventors cloned cDNA fragments into the multi-cloning site of the M13 phagemid. Competent bacterial cells were then infected with helper phages to rescue LC-antisense compounds.

[0128] A representative procedure for constructing a random gene unidirectional antisense library is as follows, with the understanding that specific embodiments and exemplifications are presented without limiting the invention in any way thereby:

[0129] (1) preparing RNA from a cell of interest, in particular, disease-related cell line or tissue;

[0130] (2) synthesizing first and second strand cDNA by reverse transcription using the RNA as the template. The second strand cDNA is synthesized from the first strand cDNA. The first strand cDNA is reverse-transcribed from purified poly(A)+mRNA using an oligo-dT primer that has a restriction enzyme site (Xho I);

[0131] (3) preparing a recombinant M13 bacteriophage by cloning the cDNA fragment into a phagemid vector. EcoR I adaptors are connected to the terminal region of the synthesized cDNA to introduce a restriction enzyme site (EcoR I). The EcoR I/Xho I fragment is produced by digesting with EcoR I and Xho I. Recombinant phages are prepared by cloning the cDNA fragments into the dephosphorylated pBluescript SK(−) vector. Phagemid vectors containing the F1 replication origin of the filamentous phage were employed for cDNA cloning depending on experimental needs. These include pUC, M13mp, pBlueScript II, pCR2.1, pGEM-f, pGL-2, pβgal, pSPORT and their derivatives;

[0132] (4) preparing bacterial transformants by introducing the recombinant M13 phagemid into competent bacterial cells. Bacterial cells such as *Escherichia coli*, and XL-10 GOLD (Stratagene) may be used. Cells may be made competent by treatment with calcium chloride; and

[0133] (5) constructing a random gene unidirectional antisense library by coinfecting the transformants with helper phage, resulting in mass production of LC-antisense compounds (FIG. 9). All phagemid vectors with the F1 (+) or F1 (−) origin are able to produce LC-antisense compounds. The pBluescript II SK(−) phagemid used in the present invention can produce LC-antisense compounds by unidirectional cloning of cDNA fragments into the cloning site cut by EcoR I/Xho I.

[0134] Random Gene Unidirectional Subtracted Antisense Library

[0135] The present invention also provides a random gene unidirectional subtracted antisense library employing the phage genome. The library may be constructed according to the following representative method, with the understanding that specific embodiments and exemplifications are presented without limiting the invention in any way thereby:

[0136] (1) preparing RNA from a cell of interest, and in particular, disease-related cell line or tissue, and synthesizing tester cDNA by reverse transcription using the RNA as template;

[0137] (2) preparing RNA from a cell of interest, and in particular, normal cell line or tissue, and synthesizing driver cDNA by reverse transcription using the RNA as template. The tester and driver cDNAs are synthesized from poly (A+) RNA of diseased and normal cells, respectively, by using oligo-dT primers with Xho I recognition site;

[0138] (3) separating the tester cDNAs into two groups, and ligating to the ends of the cDNAs belonging to one group an adaptor 1 double-stranded oligonucleotide containing a sequence recognized by a restriction enzyme, and ligating to the ends of the cDNAs belonging to the other group an adaptor 2 double-stranded oligonucleotide containing a sequence recognized by a different restriction enzyme;

[0139] (4) hybridizing (first hybridization) the driver cDNAs to both groups of tester cDNAs, thus subtracting away the commonly expressed tester cDNAs that have hybridized to the driver cDNAs.

[0140] (5) hybridizing (second hybridization) the remaining unhybridized tester cDNAs from both groups of the first hybridization by mixing the cDNAs from both groups together, and amplifying by PCR the newly hybridized cDNAs to which are bound both adaptor 1 and adaptor 2;

[0141] (6) preparing recombinant M13 phagemid by cloning the subtracted cDNA fragments into a phagemid vector;

[0142] (7) preparing transformants by introducing the recombinant M13 phagemid into competent bacterial cells; and

[0143] (8) constructing a random gene unidirectional subtracted antisense library by producing a large number of circular single-stranded molecules containing insert antisense sequence as a part of the phage genomic DNA (FIG. 10).

[0144] A subtractive hybridization method was used as described in Diachenko et al., *Proc. Natl. Acad. Sci.*, 93:6025-6030, 1996, which reference is incorporated by reference herein in its entirety.

[0145] In one embodiment of the invention, the tester cDNAs (from diseased cells) and driver cDNAs (from normal cells) are digested with Rsa I, a four-base cutting restriction enzyme, to yield blunt ends before the adaptors are ligated. For the first hybridization, an excess of driver cDNAs is added to each group of tester cDNAs. The samples are then heat denatured and allowed to anneal.

[0146] In the second hybridization between the tester cDNAs from the two groups, the two first hybridization samples are mixed together without denaturing. These new hybrids are composed of a strand from the cDNA from each group. Therefore, these hybrids are double-stranded tester molecules that have adaptors 1 and 2. The entire population of molecules is then subjected to PCR to amplify these differentially expressed sequences using primers that are complementary to both of the adaptor sequences.

[0147] For preparing the cDNA inserts, the Not I site on adaptor 1 and the Xho I site on adaptor 2 may be used for

site-specific cloning. The amplified cDNAs are digested with Not I and Xho I and ligated unidirectionally into pBluescript SK(-) vector.

[0148] Competent bacterial cells were transformed with the recombinant phagemid generally by the calcium-chloride method. The transformants were cultured and infected with helper phage to produce LC-antisense compounds. The obtained LC-antisense compounds were purified and then arrayed in multi-well plates to form an antisense library. Thus, LC-antisense molecules of differentially overexpressed genes in disease related cells were selectively obtained.

[0149] Massive Functional Genomics

[0150] The present invention also provides a high-throughput system for functional genomics using the random gene unidirectional antisense library and the random gene unidirectional subtracted antisense library discussed above. The functional genomics system of the present invention may be used to rapidly and massively search for gene function. Thus, the antisense library may be used not only for analyzing gene function but it may be used also for target validation as well as for determining the interrelationships among different gene products.

[0151] One of the advantages of using the phage genomic library for functional genomics is that it is not necessary to perform a preliminary expression profiling or to use a large number of unnecessary antisense compounds that are not specific to the type of cells used. This means that a panel of LC-antisense compounds that are specific for a particular type of cells may be used for target specific knock-down of relevant gene expression at least temporarily on a massive and parallel scale to determine genes that are responsible for a change in phenotype that is being assayed. Thus, effective antisense macroarray configurations are possible.

[0152] The LC-antisense library may be applied to a single cell type for functional assays. A panel of antisense compounds used in an antisense macroarray may be chosen based on results obtained from either a primary functional assay using the antisense library or from a conventional expression profiling or expression tracking system, such as DNA chip, SAGE, Toga or proteomics.

[0153] The LC-antisense compounds that are chosen for their function from a large antisense library may be adapted to configure an antisense macroarray. The antisense macroarray may be effectively utilized for functional comparison of the antisense compounds among different types of cells treated with the antisense compounds. Comparative functional diagnostics as well as understanding the underlying molecular mechanism of a disease may be performed by employing the inventive antisense macroarray system.

[0154] A representative massive functional genomics protocol may be as follows, with the understanding that specific embodiments and exemplifications are presented without limiting the invention in any way thereby:

[0155] (1) constructing a cDNA library using a recombinant bacteriophage vector with a single-stranded genome;

[0156] (2) identifying and selecting cDNA clones with insert sizes. Preferably, the insert size is over

500 bases. 'Cracking' method for multiple mini-scale plasmid preparation may be used;

[0157] (3) amplifying the selected clones and constructing a random gene unidirectional antisense library. Selected phagemid transformants are infected with helper bacteriophages and single-stranded phage genomic antisense compounds are subsequently harvested from culture supernatants;

[0158] (4) forming LC-antisense compound-carrier complexes;

[0159] (5) assaying for a function (primary functional assay) by positioning the LC antisense compound-carrier complexes in multi-well culture plates; and

[0160] (6) characterizing the function of genes using the antisense library and identifying the genes by DNA sequencing and subsequently comparing the obtained sequence with a sequence database. Further confirmation of gene function is carried out for the genes identified from the primary functional genomics assay (**FIG. 7**).

[0161] The cDNA library may be constructed in the same way as mentioned earlier for the construction of the random gene unidirectional and random gene unidirectional subtracted antisense libraries. Nucleotide sequence of individual members of the phage genomic antisense library has a poly(T) patch at the 5' end of the antisense insert. To prevent nonspecific binding of the poly(T) tail, masking oligos composed only of poly(A) sequences are allowed to bind to the poly(T) tail prior to cellular uptake of the antisense compounds. In this case, the masking oligos should have a minimum length of 10 bases to secure stable binding and should be phosphorothioate-capped at both ends of the molecules to improve the stability of the oligos.

[0162] Alternatively, the cDNA clones are digested with restriction enzymes or amplified with PCR using a pair of specific primers, thus eliminating the poly(A) tail. The amplified cDNA fragment is then cloned in a predetermined direction into a phagemid vector.

[0163] Recombinant phagemid clones with inserts more than the preferred size are selected using cracking or other multiple mini-scale DNA preparation methods (**FIG. 13**). A representative plasmid DNA preparation method may be as follows:

[0164] a) harvesting cells transformed with the clones from the cDNA library;

[0165] b) separating recombinant phagemid DNA from the harvested cells;

[0166] c) digesting the phagemid DNA with restriction enzymes; and

[0167] d) electrophoresing the digested phagemid DNA and confirming the size of the cDNA inserts.

[0168] The cells for the transfection of the antisense library may be chosen from cells of interest or from cells of various types of cancer, such as liver cancer, lung cancer, stomach cancer, breast cancer, bladder cancer, rectal cancer, colon cancer, prostate cancer, thyroid cancer, and skin cancer as well as cells of obesity, hair follicles of baldness, auto-immune disorders, and metabolic disorders. Cells were

seeded in wells in either suspensions or adhesive compositions depending on the cell types and properties being assayed.

[0169] It is understood that the source of the random gene unidirectional library or the host cells that may be tested need not be human. According to the principles of the invention, any source organism may be used such as, but not limited to, mammals, plants, and fungi. The host cell may be also any organism, so long as the LC-antisense compound is capable of penetrating the cell membrane or cell wall.

[0170] The LC-antisense compounds may be complexed with carriers to deliver the antisense compounds into the cells of interest. The ratio of the antisense compounds to carriers may vary based on the types of cells and types of antisense compounds that are used.

[0171] The carriers may be, but not limited to, liposomes, cationic polymers, a complex formed between cationic polymer and viral vectors, HVJ-liposomes, pronase complexes, peptides, and viral vectors. The antisense compounds may be delivered into cells either alone or complexed with the carrier composition. The LC-antisense compound-carrier complexes are mixed with cells in the multi-well plates, and the LC-antisense compounds in each well are unique in their sequence. Thus, a specific gene of interest is targeted.

[0172] The functional genomics methods described above use a defined set of chosen LC-antisense compounds applied to many types of disease cells or cells of interest. Thus, the antisense macroarray assembly is intended for functional study in a definitive and comparative manner (**FIG. 19**). The macroarray assembly may be used also for functional diagnostics, to find candidate genes for effective gene therapy, and to examine mutual relationships among genes in the diseased or abnormal cells by comparing gene functions in the cells of the same, similar or distinct lineage.

[0173] Gene functionalization assays may be performed, including observation of the morphology, growth pattern (growth promotion or inhibition) and death of the cells after the antisense compounds are applied to the cells. Such assays may be used to score parameters for the primary assays.

[0174] In addition, the present invention provides a system for gene characterization and functionalization on a massive scale using diverse types of cells treated with an antisense macroarray with a limited number of antisense compounds chosen from the phage genomic antisense library.

[0175] Cells of interest are seeded in 96-or 384-well plates and incubated for a day in a CO₂ incubator to prepare for treatment with LC-antisense compound-carrier complexes. When the 96-well plates are used in a transfection protocol, the LC-antisense compound to liposome ratio for complex formation can be either 1:3 (w/w), 1:4, or any other adequate ratio depending on the type of cells or liposomes used. In general, for the present invention, the ratio of 1:3 (W/W) was employed for efficient transfection.

[0176] To study the antisense activity in the transfected cells, the cell culture extract may be conveniently used for immunologic assays. Also, transfected cells may be used to prepare RNA, which may be used as the template for RT-PCR and Northern blotting. Several other properties such as cell morphology, death, growth patterns, and sub-

strate response may be the subject of primary functional studies. Typically, such primary functional studies make use of microscopic observations. See **FIG. 8**.

[0177] One hundred randomly selected clones were sequenced to confirm the correct orientation of the antisense inserts. Sequence data of all of the individual clones show that the antisense sequences correspond to the sense strands of their respective mRNAs (**FIGS. 12A-12C**). In addition, poly(A) tails were found at the 3' end of 96% the clones sequenced. These results demonstrate that most of the single-stranded phage genomes contain antisense sequences and that directional antisense cloning was successfully completed.

[0178] Based on the results of primary gene functionalization, further assays are carried out to confirm primary function using additional assays that utilize techniques in the fields of molecular biology, cell biology, immunology, biochemistry, animal experiments and the like. These results allow a more precise understanding of the relationships among these genes.

[0179] Functional genomics can be performed using different assays for specific genes. The following approaches may be preferably employed without limitation:

[0180] (1) measuring antisense activities to gene expression by (a) RT-PCR to detect mRNA levels, (b) Western blotting to detect protein levels, and (c) other assays for enzymatic or immunologic reactions;

[0181] (2) measuring cell growth and differentiation using MTT assay, thymidine incorporation, and colony formation on soft agarose. Factors associated with DNA replication, or chromatin activation (e.g. histone acetylase) may be measured as well;

[0182] (3) measuring apoptotic cell death, which may be scored for gene function by morphological changes, condensation of nucleus, DNA fragmentation, quantitative analysis of apoptosis, intracellular signaling for apoptosis and so on; and

[0183] (4) measuring cell cycle regulation, which may be scored by flow cytometry analysis, activities of factors involved in cell cycle progression or pause, and by complex formation between factors involved in cell cycle.

[0184] In addition to the above methods, other methods for functional genomics using antisense inhibition techniques include assays using molecular biological, biochemical, and physiological changes in vitro and in vivo.

[0185] The phage vector allows easy production of the long single-stranded sequence that encompasses the antisense sequence with high sequence fidelity. The new antisense molecules, even with their unconventionally long length, exhibited good sequence specificity in eliminating expression of target mRNA. Without being bound by any particular theory or mechanism of action of the antisense nucleic acid, it is thought that once a small portion of the antisense sequence binds to its complementary sequence, the antisense sequence zips through the entire length of the complementary target sequence. The lengthy duplex formed between the antisense DNA and sense RNA is then much more stably maintained as a substrate for RNaseH activity.

[0186] Another reason for the advantageous binding of the inventive antisense molecule may be that there may exist a higher chance for the long antisense molecule to bind to a target site that is structurally exposed. Messenger RNA tends to form extensive secondary and tertiary structures within its own sequence and by interaction with RNA binding proteins in the cell cytoplasm. Finding an open target site for an antisense molecule is critical for successful antisense activity. With its long length, the phage genomic antisense molecule has to have some sequence that can access exposed complementary sequences of target mRNA, thus improving the chances for target mRNA ablation.

[0187] Antisense Molecular Therapy

[0188] The inventive LC-antisense compounds are effective therapeutic agents against various types of cancer, viral infection, immunologic disorders, metabolic disorders and other human diseases in which modulation of gene expression can be beneficial to intervene in disease initiation and progression.

[0189] The principles of the antisense molecules of the invention may be applied to any target gene of interest. While TNF- α and NF- κ B specific LC-antisense compounds are disclosed as examples of the antisense molecule of the invention, the antisense molecule of the invention may be made against any gene of interest. In fact, the LC-antisense compounds of the invention were significantly more stable to nucleases and were effective in target ablation. Exemplified sequence specific reduction of the TNF- α and NF- κ B target genes supports the broad utility of an antisense molecular therapy method. Thus, the antisense molecule of the invention may be used to bind to any endogenously expressed target transcript from any source.

[0190] Antisense activity was also examined at the protein level to ensure correlation of both target mRNA and protein elimination. Administration of TNF α -M 13AS was found to significantly reduce rat TNF- α secretion in cell culture media, confirming effective antisense activity. In contrast, control phage genomic compounds (single-stranded circular molecules without an antisense insert) exhibited only a mild reduction in TNF- α secretion. The slight decrease of TNF- α secretion by the addition of control antisense molecule can be explained, in part, by the cytotoxicity of free cationic liposomes deposited inside endosomes. Cells treated with cationic liposomes alone exhibited lower viability than cells with LC-antisense compound-liposome complex.

[0191] In therapeutic applications, the large circular nucleic acid molecules can be formulated for a variety of modes of administration, including oral, topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., latest edition. The active ingredient that is the antisense molecule is generally combined with a carrier such as a diluent of excipient which may include fillers, extenders, binding, wetting agents, disintegrants, surface-active agents, erodible polymers or lubricants, depending on the nature of the mode of administration and dosage forms. Typical dosage forms include tablets, powders, liquid preparations including suspensions, emulsions and solutions, granules, and capsules.

[0192] Certain of the large circular nucleic acid compounds of the present invention may be particularly suited

for oral administration which may require exposure of the drug to acidic conditions in the stomach for up to about 4 hours under conventional drug delivery conditions and for up to about 12 hours when delivered in a sustained release form. For treatment of certain conditions it may be advantageous to formulate these antisense compounds in a sustained release form.

[0193] Systemic administration of the large circular nucleic acid molecules may be achieved by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, bile salts and fusidic acid derivatives for transmucosal administration. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through use of nasal sprays, for example, as well as formulations suitable for administration by inhalation, or suppositories.

[0194] The large circular nucleic acid molecule of the present invention can also be combined with a pharmaceutically acceptable carrier for administration to a subject. Examples of suitable pharmaceutical carriers are a variety of cationic lipids, including, but not limited to N-(1,3-dioleoyloxy)propyl)-n,n,n-trimethylammonium chloride (DOTMA) and dioleoylphosphatidyl ethanolamine (DOPE). Liposomes are also suitable carriers for the antisense molecules of the invention. Another suitable carrier is a slow-release gel or polymer comprising the claimed antisense molecules.

[0195] The large circular nucleic acid molecules may be administered to patients by any effective route, including intravenous, intramuscular, intrathecal, intranasal, intraperitoneal, intratumoral, subcutaneous injection, in situ injection and oral administration. Oral administration may require enteric coatings to protect the claimed antisense molecules and analogs thereof from degradation along the gastrointestinal tract. The large circular nucleic acid molecules may be mixed with an amount of a physiologically acceptable carrier or diluent, such as a saline solution or other suitable liquid. The antisense molecules may also be combined with other carrier means to protect the nucleic acid molecules or analogs thereof from degradation until they reach their targets and/or facilitate movement of the antisense molecules or analogs thereof across tissue barriers.

[0196] In one embodiment, the large circular nucleic acid molecules are administered in amounts effective to inhibit cancer or neoplastic cell growth. In other embodiments, the antisense molecule may be used to treat viral infections, such as, but not limited to herpes, human papilloma virus (HPV), HIV, small pox, mononucleosis (Epstein-Barr virus), hepatitis, respiratory syncytial virus (RSV) and so on. In addition, metabolic diseases, such as, but not limited to, phenylketonuria (PKU), primary hypothyroidism, galactosemia, abnormal hemoglobins, types I and II diabetes, obesity and so on are also targets. The inventive antisense molecule may be used to treat other diseases such as immunologic diseases including such diseases as, but not limited to, Sjogren's Syndrome, antiphospholipid syndrome, immune complex diseases, Purpura, Schoenlein-Henoch, immunologic deficiency syndromes, systemic lupus erythematosus, immunodeficiency, rheumatism, and so on.

[0197] The actual amount of any particular large circular nucleic acid molecule administered will depend on factors such as the type and stage of the disease or infection, the toxicity of the antisense molecule to other cells of the body, its rate of uptake by the cells, and the weight and age of the individual to whom the nucleic acid molecule is administered. An effective dosage for the patient can be ascertained by conventional methods such as incrementally increasing the dosage of the antisense molecule from an amount ineffective to inhibit cell proliferation to an effective amount. It is expected that concentrations presented to the diseased cells may range from about 10 nM to about 30 μ M will be effective to inhibit gene expression and show an assayable phenotype. Methods for determining pharmaceutical/pharmacokinetic parameters in chemotherapeutic applications of antisense molecules for treatment of cancer or other indications are known in the art.

[0198] The large circular nucleic acid molecules are administered to the patient for at least a time sufficient to have a desired effect. To maintain an effective level, it may be necessary to administer the antisense nucleic acid molecules several times a day, daily or at less frequent intervals. For cancer cells, antisense molecules are administered until cancer cells can no longer be detected, or have been reduced in number such that further treatment provides no significant reduction in number, or the cells have been reduced to a number manageable by surgery or other treatments. The length of time that the antisense molecules are administered will depend on factors such as the rate of uptake of the particular molecule by cancer cells and time needed for the cells to respond to the molecule.

[0199] The following examples are offered by way of illustration of the present invention, and not by way of limitation.

EXAMPLES

EXAMPLE 1

Construction of LC-Antisense Compounds Using M13 Bacteriophage

[0200] Experiments were carried out to determine whether the circular phage genome of M13 bacteriophages (phage) can harbor an antisense sequence as a part of its genome and whether these new antisense molecules can overcome the problems associated with synthesized forms of antisense oligonucleotides. Production of recombinant M13 phage was carried out by infecting M13K07 helper phages into bacterial cells that were already transformed with pBluescript KS (–) phagemid (Jupin et al. *Nucleic Acid Res.*, 23, 535-536 (1995)). We utilized the F1 replication origin of the phagemid to generate single-stranded circular phage genome containing either antisense or sense sequence for a target gene. In the case of the gene encoding rat TNF- α , the entire cDNA of the gene was placed into pBluescript KS (–) vector to produce the antisense sequence (FIG. 1).

[0201] The antisense sequence in the single-stranded genomic DNA was confirmed by DNA sequencing using T7 sequencing primers (FIG. 2). Both the 5' and 3' flanking sequences of the TNF- α antisense insert were shown to be those of the phagemid vector. The insert sequence corresponded with that of TNF- α mRNA, demonstrating that the

antisense sequence was present. The circular phage genome containing the antisense sequence for TNF- α and NF- κ B were designated as TNF- α -M13AS and NF- κ B-M13AS, respectively.

[0202] 1. mRRNA Induction and Cloning of Genes Encoding Rat TNF- α and Human NF- κ B

[0203] Rat TNF- α expression was induced with lipopolysaccharide (LPS, 30 μ g/ml) in WRT7/P2 cells. Cells at 1×10^6 cells/well were seeded in each well of a 48-well plate and were treated with LPS for 4 to 24 hours. Cells were harvested at desired time points to examine the amounts of mRNA. The LPS incubation time by which TNF- α expression was induced at the highest level was chosen for further experiments. The highest level of rat TNF- α expression was determined 6 hours after LPS treatment.

[0204] Rat TNF- α cDNA was obtained from the amplified cDNA fragments as described above. The RT-PCR fragment (708 bp) of TNF- α that comprises the entire coding sequence was amplified with a pair of PCR primers: 5'-GATCGTCGACGATGAGCACAGAAAGCATGATCC-3' (SEQ ID NO:1), and 5'-GATCGAATTCGTCACAGAGCAATGACTCCAAAG-3' (SEQ ID NO:2). The rat TNF- α cDNA fragment was cloned into the multiple cloning site of pBluescript KS (–) vector using Sal I and EcoR I restriction sites in the same direction as the lacZ gene (FIG. 1).

[0205] Similarly, cDNA fragments of the NF- κ B gene was amplified with a pair of PCR primers and cloned into the EcoRV site of pBS-KS (+) vector after blunting the ends. Amplified cDNA fragments were always confirmed with both restriction digestion and DNA sequencing.

[0206] In detail, THP1 cells derived from leukocytic monocytes which were transfected with NF- κ B-M13AS, NF- κ B-M13SE or M13SS complexed with liposomes in a ratio of DNA to liposome ratio of about 1:4 (w/w) and cultured. One day after lipofection, cells were stimulated with PMA (160 nM) for 6 hours. Total RNA was isolated and subjected to RT-PCR using a pair of primers: 5'-GATCGTCGACGCGCCACCCGGCTTCAGAATGGC-3' (SEQ ID NO:3) and 5'-GATCGAATTCGGTGAAGCTGCCAGTGCTATCCG-3' (SEQ ID NO:4). The PCR product was used in Southern blot analysis using a 25 mer oligonucleotide probe of 5'-CTTCCAGTGCCCCCTCCTCCACCGC-3' (SEQ ID NO: 5).

[0207] 2. Construction of Large Circular Nucleic Acid Molecules Employing a Phagemid Vector and the M13K07 Helper Bacteriophages

[0208] (1) Construction of Single-Stranded Bacteriophage Genome Harboring either Sense or Antisense Sequences

[0209] Large circular nucleic acid molecules that contain an antisense region specific to the target genes were constructed according to standard cloning procedure (Sambrook et al., *Molecular Cloning*, 1989). Competent bacterial cells (XL-1 Blue MRF') containing the pBS-KS (+) or (–) phagemid with the appropriate cDNA were infected with helper bacteriophage M13K07 (NEB Nucleic Acids, USA). The orientation of the cloned cDNA in the phagemid vector determines which of the sense or antisense sequence will be produced. 20% polyethylene glycol (PEG 8000) was added to the supernatant of an overnight culture of helper phage infected cells grown in 2 $\times$ YT. The bacteriophage precipitate

was resuspended in TE (pH 8.0), and phage genomic DNA was isolated by phenol extraction and ethanol precipitation.

[0210] (2) Purification of the Phage Genomic Antisense Molecules

[0211] Purification of phage genomic antisense molecules from the residual genomic DNA of helper bacteriophage and host bacterial cells was carried out either with 0.8% low melting point (LMP) agarose gel for small scale purification or with gel filtration column chromatography (1.0x50 cm) for large scale purification. The column resin for gel filtration was superfine Sephacryl™ S-1000 (molecular cutoff: 20,000 bp) (Amersham Pharmacia Biotech AB, Sweden), and was packaged and equilibrated with 50 mM Tris-HCl buffer containing 0.2 M NaCl (pH 8.3). The starting volume of the antisense molecules was adjusted to 5% of the gel void volume and DNA elution was carried out with the same buffer used for resin equilibration (flow rate: 0.3 ml/min). Samples were UV scanned at 260/280 nm with a dual UV detection system and were collected every 5 min during elution. Sample fractions were washed and precipitated with 70% cold ethanol and were resuspended in distilled ultra-pure water and PBS (phosphate-buffered saline) for subsequent experiments. The purified antisense molecules were tested for quantity and purity on a 1% agarose gel. Control sense molecules were constructed with the TNF- α cDNA fragment cloned in pBS-KS (+), in the opposite orientation of the lacZ gene in the vector. Single-stranded molecules of either sense or antisense were confirmed for sequence integrity by employing the T7 primer for sequencing. DNA sequencing was carried out with an automated DNA sequencer (**FIG. 2**).

[0212] EXAMPLE 2

Structural Analysis and Stability Test of the Phage Genomic Circular Antisense Molecules

[0213] 1. Single-Stranded Circular TNF- α Antisense Molecules

[0214] The fact that the antisense molecules are single stranded, circular and stable was tested in the following manner. 1 μ g LC-antisense molecules containing antisense region targeted to the gene encoding TNF- α were treated with Xho I (10 U/ μ g DNA), Exonuclease III (160 U/ μ g DNA), or S1 nuclease (10 U/ μ g DNA) at 37° C. for 3 hrs, and subjected to phenol extraction, ethanol precipitation and gel electrophoresis on a 1% agarose gel to study their stability as well as digestion patterns.

[0215] TNF- α -M13AS was tested for its circular structure and stability to nucleases. The LC-antisense molecules were expected to be stable to exonucleases because of their closed circular structure. When TNF- α -M13AS was incubated with the endonuclease Xho I and exonuclease III, the antisense molecules were found to be largely intact even after a 3 hour incubation with these nucleases (**FIG. 3A**). In contrast, when Xho I was added to the double-stranded replication form of the recombinant M13 phage DNA, the DNA was, as expected, completely digested by the combination of the restriction enzyme and exonuclease III. The single-stranded TNF- α -M13AS was also completely digested by S1 nuclease, a nuclease that is specific for single-stranded DNA. Thus, it was confirmed that TNF- α -M13AS was shaped as a single-stranded circular molecule.

[0216] 2. Stability Test for TNF- α -M13AS

[0217] For the stability test, 1 μ g of antisense molecules was added alone or after complex formation with liposomes in a ratio of DNA:liposome of about 1:3 (w/w). A non heat inactivated 30% FBS solution was added to the antisense-liposome complex and incubated at 37° C. for varying time periods for up to 48 hours. After incubation with FBS and the nucleases, antisense DNA was extracted with chloroform, precipitated with ethanol and electrophoresed on a 1% agarose gel.

[0218] Phage genomic antisense molecules were also found to be stable since their structural integrity was largely preserved after incubation with serum. When TNF- α -M13AS was combined with cationic liposomes, a large fraction of the antisense molecules remained intact after extended incubation in fetal bovine serum (FBS). In fact, TNF- α -M13AS remained intact even after a 24 hour incubation with 30% FBS (**FIG. 3B**). The results suggest that the phage genomic antisense molecules may be further stabilized during in vivo application by forming complexes with liposomes.

EXAMPLE 3

Effective and Specific Elimination of Rat TNF-Alpha Expression by TNF-Alpha-M13AS

[0219] The antisense activity of TNF- α -M13AS was tested. TNF- α -M13AS contains a long antisense sequence that includes nonspecific antisense phagemid vector sequences and an antisense region specific to rat TNF- α mRNA. The fact that the phage genomic antisense molecules have a large amount of nonspecific sequences necessitates a thorough analysis of target specificity of the antisense activity. In order to determine whether phage genomic antisense molecules act specifically to eliminate target gene expression, multiple control genes were used to compare levels of mRNA ablation.

[0220] 1. Cell Cultures

[0221] Monocytic mouse cell line WRT7/P2 and human cell line THP-1 were maintained in either RPMI 1640 or EMEM (JBI, Korea) supplemented with 10% heat-inactivated FBS (JBI, Korea), 100 μ g/ml penicillin and 100 μ g/ml streptomycin. Cells were cultured in a CO₂ (5%) incubator at 37° C. and carefully maintained to avoid over growth. Cell media was exchanged with fresh culture media the day before lipofection (16 hours) and tested for cell viability with 0.4% trypan blue staining on the day of experiments.

[0222] 2. Transfection of TNF- α -M13AS Complexed with Liposomes

[0223] Cationic liposomes, such as Lipofectamine™, Lipofectamine 2000™ or Lipofectamine Plus™ (Life Technologies, USA) were mixed with either antisense molecules or sense control molecules. These liposome-DNA complexes were mixed with OPTI-MEM (Life Technologies, USA), and were then added to cells according to the protocol suggested by the manufacturer.

[0224] Lipofection details are as follows. Cells were cultured in RPMI 1640 or EMEM supplemented with 10% FBS and were washed twice with OPTI-MEM 30 minutes prior to lipofection. Cells were seeded in a 48-well plate (1x10⁵

cells/well) in 200 μ l of culture media. Antisense molecules were mixed with cationic liposomes in a ratio of about 1:3 (w/w) and added to cells for transfection. Cells were incubated for 6 hours at 37° C. in serum-free media. Following the lipofection, 2 $\times$ FBS and antibiotics were added to the culture medium and incubated further for 18 hrs at 37° C. Rat TNF- α expression was induced with LPS (30 μ g/ml). Cells were used for the preparation of RNA, and culture supernatant was tested for the presence of IL-10 with Enzyme Linked Immuno-Sorbent Assay (ELISA).

[0225] 3. Detection of Transcription with RT-PCR

[0226] RNA preparation was carried out with Tri reagent™ (MRC, USA) according to the protocol recommended by the manufacturer. Cells harvested from each well were mixed with 1 ml Tri Reagent and 200 μ g chloroform for RNA purification. Purified RNA was subjected to RT-PCR in a 50 μ g reaction volume by using the Access™ RT-PCR kit (Promega, USA). In a PCR tube were added purified RNA, a pair of primers: 5'-CATCTCCCTCCG-GAAAGGACAC-3' (SEQ ID NO:6) and 5'-CGGATGAACACGCCAGTCGC-3' (SEQ ID NO:7), AMV reverse transcriptase (5 U/ μ l), Tfl DNA polymerase (5 U/ μ l), dNTP (10 mM, 1 μ l) and MgSO₄ (25 mM, 2.5 μ l). Reverse transcription and polymerase chain reaction were sequentially carried out in a thermal cycler (Hybaid, UK). Synthesis of the first strand cDNA was carried out at 48° C. for 45 min and subsequent DNA amplification was carried out in 30 repetitive cycles, at 94° C. for 30 sec (denaturation), 59° C. for 1 min (annealing), and 68° C. for 2 min (polymerization). PCR product was confirmed on a 1% agarose gel, and quantitative analysis of the amplified DNA was performed with Alphasizer 1220, a gel documentation apparatus (Alpha Inno-Tech corporation, USA).

[0227] 4. Southern Blotting

[0228] Probes for Southern hybridization were prepared with ECL (enhanced chemical luminescence) oligo-labeling and detection system (Amersham Life Science, UK). RT-PCR products were run on a 1% agarose gel and transferred onto a nylon membrane in 0.4 M NaOH solution. An oligonucleotide probe for TNF- α was a 22 mer: 5'-GATGAGAGGGAGCCCATTTGGG-3' (SEQ ID NO:8), and an oligonucleotide probe for NF- κ B was a 25 mer: 5'-CTTC-CAGTGCCCCCTCCTCCACCGC-3' (SEQ ID NO:5).

[0229] Oligonucleotide probes of 100 pmol were mixed with fluorescein-11-dUTP, cacodylate buffer and terminal transferases, and were incubated at 37° C. for 70 min for ECL labeling. Probe hybridization to a nylon membrane with transferred DNA was carried out in a 6 ml hybridization buffer (5 $\times$ SSC, 0.02% SDS, liquid block) at 42° C. for 14 hrs. The nylon membrane was washed twice in 5 $\times$ SSC containing 0.1% SDS and once in 1 $\times$ SSC containing 0.1% SDS, at 45° C. for 15 min for each washing. The membrane was incubated with an antibody conjugated to HRP anti-fluorescein for 30 min, followed by incubation with ECL detection reagent for about 5 min before exposure to an X-ray film.

[0230] To test the specific activity of TNF α -M13AS, 0.5 μ g (1.4 nM) of the antisense molecules were complexed with 1.5 μ g of cationic liposome and were added to 1 $\times$ 10⁵ cells of a monocytic cell line, WRT7/IP2. The cells were then induced for TNF- α expression by LPS treatment. When the

cells were treated with TNF α -M 13AS, the induction level of TNF- α mRNA was significantly reduced. In contrast, when cells were treated with either TNF α -M13SE (the sense strand of TNF- α) or M13SS (single-stranded phage genome without the antisense insert) they did not show much reduction of TNF- α mRNA (FIGS. 4A and 4C). RT-PCR band of TNF- α was confirmed by Southern hybridization using a probe that binds to the middle of the amplified DNA fragments.

[0231] TNF α -M13AS contains the rat TNF- α antisense sequence as well as antisense sequences of the β -galactosidase (LacZ) and the β -lactamase (Amp) genes, harboring a total of 3.7 kb single-stranded circular genome. The TNF- α specific antisense portion is about 708 bases long. Thus, the TNF- α specific antisense sequence in TNF α -M13AS is itself very long when compared with conventional synthetic antisense molecules of some 20 or 30 nucleotides. This is significant because it has been generally believed in the art that as the antisense molecule is lengthened, its sequence specificity declines. Further confirming tests were carried out to show that the antisense activity of TNF α -M 13AS is indeed sequence specific.

[0232] In order to demonstrate sequence specific antisense activity, three different genes were examined for mRNA levels after lipofection of TNF α -M13AS. These were β -actin, GAPDH (glyceraldehyde 3-phosphate dehydrogenase), and IL-1 β (interleukin-1 β). Expression of these genes was not affected by lipofection of TNF α -M13AS (FIGS. 4A-4C).

[0233] Dose response of TNF α -M13AS in its antisense activity was also examined. When TNF α -M13AS was used at a concentration of 0.01 μ g (0.03 nM), TNF- α expression was only slightly reduced. At a concentration of 0.05 μ g (0.14 nM), TNF- α expression was partially eliminated. When the amount of TNF α -M13AS was increased to 0.1 μ g (0.28 nM), TNF- α mRNA was found to be completely abolished. These results show that TNF α -M13AS is effective for the elimination of target mRNA using a much smaller amount than conventionally used antisense molecules.

EXAMPLE 4

Expression Patterns of Rat TNF α Protein

[0234] Quantitation of target proteins after antisense treatment was examined with either ELISA or Western blotting method. For the ELISA assay, cell culture supernatant was diluted 50 fold and added to an ELISA plate coated with antibody against TNF- α . Biotinylated secondary antibody to anti TNF- α was added into each well of the ELISA plate and incubated at room temperature for 90 minutes. After three washings, streptavidin-peroxidase was added, and incubated for 45 minutes. The plate was washed four times to remove unbound streptavidin-peroxidase, and chromogen was added. After a 20 min incubation for color development, optical density was measured at 450 nm.

[0235] WRT7/P2 cells were lipofected with TNF α -M13AS, and TNF- α secreted from the transfectants was measured using the ELISA assay. Similar to the level of reduction of endogenous TNF- α mRNA, TNF- α protein in the cell culture supernatant was also reduced by more than 90% after administering TNF α -M13AS (FIG. 5). However,

neither of the control antisense molecules, TNF α -M13SE (containing the sense strand of the TNF- α gene) or M13SS, reduced TNF- α expression in WRT7/P2 transfectants. These results demonstrate that TNF α -M13AS was effective in both the elimination of TNF- α mRNA and subsequent disappearance of TNF- α from the transfectants.

EXAMPLE 5

Effect of NFKAPPAB-M13as on Human NFKAPPAB Transcription

[0236] Observing the effectiveness of TNF α -M13AS, experiments were carried out to determine whether phage genomic antisense compounds specifically directed to other genes block the expression of another gene, such as NF- κ B. LC-antisense compound to NF- κ B (NF κ B-M13AS) was produced and tested in THP-1 cells for efficient antisense activity. NF κ B-M13AS was also complexed with liposomes and was added to the cells in increasing amounts. When 0.05 μ g (0.14 nM) of NF κ B-M13AS was added to THP-1, NF- κ B mRNA was reduced by about 70%. When the amount of NF κ B-M13AS was increased to 0.1 μ g (0.28 nM) and to 0.2 μ g (0.56 nM), NF- κ B mRNA was eliminated by more than 90%. In contrast, cells that were treated with either NF κ B-M13SE (phage genomic DNA with the sense sequence of NF- κ B) or with M13SS, NF- κ B expression was not much affected (FIGS. 6A-6B).

EXAMPLE 6

Construction of Unidirectional Subtracted Liver Antisense Library

[0237] 1. Construction Of Unidirectional Subtracted Liver cDNA Library

[0238] To raise the efficiency for searching disease-related genes through gene functionalization, a random antisense library was constructed that included phage genomic antisense molecules that contain genes that are differentially and specifically expressed or overexpressed in cancer tissue but not in normal tissue (FIG. 10). For differential cDNA cloning of overexpressed genes in liver cancer, the present inventors used a subtractive hybridization method as described in Diachenko et al., *Proc. Natl. Acad. Sci.*, 93: 6025-6030, 1996, which reference is incorporated herein by reference in its entirety.

[0239] Total RNA was prepared from normal and cancerous liver tissue using Tri Reagent™ (MRC, USA) according to the protocol recommended by the manufacturer. Briefly, tissue samples were washed in phosphate-buffered saline solution and sliced into smaller pieces, and homogenized for 10 minutes in an optimal volume of Tri Reagent™.

[0240] Poly(A)+mRNA was purified using poly(A) Quick mRNA Isolation Kit (Stratagene, USA) according to manufacturer's instructions. Purified poly(A)+mRNA was used as template for the synthesis of the first strand cDNA. To clone the cDNA inserts directionally, the Hind III recognition sequence was replaced with a Xho I recognition sequence in the cDNA synthesis primer: 5'-TTTTGTACCTC-GAGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3' (SEQ ID NO:9) supplied with a cDNA subtraction kit (PCR-Select™ cDNA Subtraction Kit, Clontech Laboratories, Inc.,

USA) and synthesized the first strand cDNA followed by the second strand cDNA using the standard protocol supplied by the manufacturer.

[0241] The tester cDNA (from cancer cells) and driver cDNA (from normal cells) prepared by the above mentioned procedure were digested with Rsa I, a four-base cutting restriction enzyme that yields blunt ends. The tester cDNAs were then subdivided into two groups, and cDNAs from each group were separately ligated to group-specific cDNA adaptors.

[0242] Adaptor 1: 5'-ACCTGCCCCGG-3' (SEQ ID NO:10), which is complementary to a portion of 5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCCGGGCAGGT-3' (SEQ ID NO:11).

[0243] Adaptor 2: 5'-ACCTCGGCCG-3' (SEQ ID NO:12), which is complementary to a portion of 5'-CTAATACGAC TCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3' (SEQ ID NO:13).

[0244] Two hybridization reactions were performed, which were followed by suppression PCR to amplify differentially expressed sequences. Differentially expressed sequences were amplified by PCR method using a pair of primers: 5'-TCGAGCGGCCGCCCCGGGCAGGT-3' (SEQ ID NO:14) and 5'-AGCGTGGTCG CGGCCGAGGT-3' (SEQ ID NO:15). This procedure eliminated background signal. The subtracted cDNA pool was cloned into the multiple cloning site of pBluescript (pBS) SK(-) vector using Not I and Aho I restriction sites in the same direction as the LacZ gene. The CDNA plasmid library was transformed into Epicurian Coli® XL-10 Gold Ultracompetent Cells (Stratagene, USA) by the calcium-chloride method.

[0245] The quality of the obtained cDNA library was tested. First, to determine the total number of primary transformants, 1 μ l and 10 μ l aliquots from 1 ml pilot transformants were plated on LB-ampicillin agar plates, separately. Primary library size was determined as 2×10^4 cfu/ml.

[0246] Second, for determining the percentage of vectors with inserts and the average size of the inserts, 40 randomly selected recombinant phagemid clones were purified and digested. And it was determined that 97.5% of the tested transformants had CDNA insert, and the average insert size was approximately 300 bp (FIG. 11).

[0247] Third, to determine whether directional cloning was successful, we sequenced 100 randomly selected clones from the 5' end of the (+) strand of the cDNA in pBS SK(-) phagemid by employing T3 primer, and confirmed that 96% of recombinant phagemids had inserts in the intended orientation.

[0248] 2. Selection Of Transformants Containing Phagemids With Inserts Of Preferred Size

[0249] Transformants containing phagemids that have cDNA inserts of more than about 500 bases were selected by the so-called 'cracking' method.

[0250] Each single colony was seeded in each well of 96 deep well plates and incubated in 1 ml of LB liquid media containing 50 μ g/ml of ampicillin for 20 hours at 37° C. in a rapidly shaking incubator. 300 μ l of 1 ml overnight culture was centrifuged, and cell pellets were vortexed vigorously

for 30 seconds in 40 μ l of gel loading buffer (0.25% bromophenol blue, 30% glycerol) and 14 μ l of phenol-chloroform mixture (25:24).

[0251] After centrifugation of each sample for 10 minutes at 12,000 rpm at room temperature, 8 μ l of supernatant was loaded on a 1.0% agarose gel. As a control, two kinds of transformants containing pBS SK(-) phagemid with and without a 500 bp cDNA insert were cracked together and electrophoresed on an agarose gel simultaneously (FIG. 13). Selected transformants containing 1,200 of 9,600 clones that had been cracked and which contained cDNA insert larger than 500 bp were arranged in 96-well plates and cultured. The cells were further preserved in a -70° C. deep freezer as glycerol stocks.

[0252] 3. Making Liver-Specific Unidirectional Subtracted LC-Antisense Compounds

[0253] Bacterial culturing and purification steps for making a unidirectional subtracted liver antisense library were performed as follows. Competent bacterial cells containing pBS SK(-) phagemid with a cDNA insert were plated on LB agar plates containing 50 μ g/ml of ampicillin and 50 μ g/ml of tetracycline and incubated at 37° C. for 16 hours. Isolated single colonies that were seeded in each well of 96-deep well plate, were aliquotted with 1.5 ml 2 $\times$ YT liquid media (tryptone 16 g, yeast extract 10 g, NaCl 10 g per 1000 ml) containing 50 μ g/ml ampicillin, and precultured for 7 hrs at 37° C. with vigorous shaking. To produce LC-antisense compounds from each phagemid, 20 μ l of the preculture was multi-channel pipetted to the wells prefilled with 1.4 ml 2 $\times$ YT liquid media free of ampicillin, but which also contained 9 μ l of helper bacteriophage M13K07 (NEB Nucleic Acids, USA).

[0254] After a 1 hour incubation, 4.2 μ l of 50 μ g/ml kanamycin was added and cultured for 12 hours under the same conditions described above. The infection was carried out in triplicate for each clone to maximize the yield of antisense molecules in a single purification step.

[0255] For high-throughput massive production of single-stranded LC-antisense molecules, 20% polyethylene glycol (PEG 8000) was added to culture supernatant of the overnight culture using QIAprep 96 M13 Kits (Qiagen, German). Purification steps were performed with a QIAVAC Vacuum Manifold (Qiagen, German) following manufacturer's instructions.

[0256] Purified LC-antisense molecules were run together with control molecules derived from pBS SK(-) phagemid without a cDNA insert on a 1% agarose gel to test the quantity and purity of the antisense molecules (FIG. 14).

[0257] After confirming adequate purification of the phage genomic antisense compounds by gel electrophoresis, the random gene subtracted liver antisense library that includes approximately 1200 member clones was arrayed in thirteen 96-well plates.

EXAMPLE 7

Lipofection of Liver-Specific Random Gene Unidirectional Subtracted Antisense Library Into Liver Cancer Cells

[0258] This is an example of applying the antisense library to determine genes that are involved in the disease process

of a particular cell line. By the principle that specific binding of antisense library molecules to the complementary mRNA sequence can inhibit the expression of the target gene, the present inventors first screened LC-antisense compounds affecting the growth of liver cancer cells by lipofection of the liver-specific random gene unidirectional subtracted antisense library into a liver cancer cell line.

[0259] A liver cancer cell line, HepG2, was obtained from Korean cell line bank (KCLB, Korea). The cell line was maintained in DMEM media (JBI, Korea) supplemented with 10% heat-inactivated FBS (JBI, Korea), 100 μ g/ml of penicillin and 100 μ g/ml of streptomycin.

[0260] After washing the cells twice with OPTI-MEM (Life Technologies, USA), 7 $\times$ 10³ cells were seeded in each well of the thirteen 96-well plates in 100 μ l of optimal culture media supplemented with 10% FBS. The cells were incubated for 12-18 hours at 37° C. in a 5% CO₂ incubator. 0.1 μ g of each LC-antisense molecule that was to be transferred into the thirteen 96-well plates was complexed with 0.3 μ g of cationic liposomes, and the LC-antisense compound-carrier complex was added to the cultured cells. Cell media were changed with fresh media 24 hours after transfection and incubated for 4 more days.

[0261] To compare the effects of the LC-antisense molecules on cell proliferation, identical quantities of carrier alone and control DNA-carrier complexes were also added to the cells in a different 96-well plate and assayed simultaneously. Control DNA was a large circular phage genomic DNA without a cDNA insert.

EXAMPLE 8

Screening for Genes Critical for Growth of Liver Cancer Cells

[0262] In order to screen for genes involved in the growth of liver cancer cells, light microscopy, MTT reduction assay and [3H]-thymidine incorporation assays were performed. Growth inhibition of liver cancer cells using LC-antisense compounds was first confirmed by light microscopy (original magnification, $\times$ 200) at 4 days after transfection with the LC-antisense compound-carrier complex (FIGS. 15A-15I).

[0263] For the MTT reduction assay, at 4 days after the transfection of LC-antisense compounds, cell culture media was replaced with 50 μ l of fresh media. 25 μ l of 5 mg/ml MTT reagent (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide, in phosphate-buffered saline, SIGMA, USA) was added to each well of the 96-well plates by multi-channel pipetting, followed by incubation at 37° C. for 4 hours. 150 μ l of isopropanol containing 0.1 N HCl was added to the cells and incubated at room temperature for 1 hour. Absorbance was measured at 570 nm with Spectramax 190™ (Molecular Devices, USA) to score the amount of cells that survived.

[0264] The percentage of growth inhibition was calculated using the following formula:

[0265] Percentage of growth inhibition = 1 - (Absorbance of an experimental well / Absorbance of a control well) $\times$ 100.

[0266] The percentage of growth inhibition in the experimental wells treated with LC-antisense compound-carrier complex, and the control wells that were either sham treated,

treated with carrier alone, or were treated with control DNA-carrier complexes, were all measured by optical density, and the recorded absorbance readings compared with each other (FIGS. 16). Single-stranded DNA without an insert that was purified from bacterial culture was used as control DNA.

[0267] From 1,200 antisense molecules screened in this manner, 153 (~12.8%) independent LC-antisense molecules displayed about 30~90% growth-inhibition. The results indicate that these 153 functionally identified genes promote the proliferation of liver cancer cells.

[0268] The liver cancer cell growth inhibiting activity of the above-mentioned 153 LC-antisense compounds was confirmed by MTT assay and [³H]-thymidine incorporation assay performed on a configured LC-antisense compound macroarray assembly (FIGS. 17A-17D and FIGS. 18A-18D). For the [³H]-thymidine incorporation assay, 0.5 μ Ci of [³H]-thymidine (2.0 Ci/mmol, Amersham Pharmacia Biotech) was added to cells 24 hours after transfection, and the cells were incubated at 37° C. in a CO₂ incubator. After 4 days, the cells were treated with trypsin (Life Technology, USA) and harvested on a glass microfiber filter (GF/C Whatman, Madistone, Kent, UK). The filter was washed with cold phosphate-buffered saline, and then treated with 5% trichloroacetic acid and absolute alcohol, successively. [³H]-thymidine incorporation was measured by a liquid scintillation counter in a mixture solution containing toluene, Triton X-100, 2,5-diphenyloxazole and 1,4-bis[2-(5-phenyloxazolyl)]benzene. The percentage of growth inhibition was calculated using the following formula:

[0269] Percentage of growth inhibition=1-(cpm of an experimental well/cpm of a control well)×100.

EXAMPLE 9

Identification of Genes Critical for Growth of Liver Cancer Cells

[0270] It is possible that several copies of a particular cDNA may be present within a random gene unidirectional subtracted antisense library. Therefore, in order to determine the uniqueness of the identified 153 clones, these clones were individually sequenced.

[0271] Purified recombinant phagemids obtained by alkaline lysis method were sequenced from the 5' end of the (+) strand of the cDNA region by employing T3 primer. The

elucidated sequence for each clone was compared with GenBank database, and 80 unique genes (unigenes) were identified. Table 1 shows some of the functionally identified genes involved in the growth of liver cancer cells. Surprisingly, 44 of the 80 unigenes were of unknown function.

EXAMPLE 10

Functional Profiling of Antisense Compounds Against Disease Cells in a Macroarray Configuration

[0272] To study the antisense activity profile of the 80 genes obtained from the unidirectional subtracted liver antisense library, cultured cells of Hep3B (liver cancer), NCI-H1299 (non-small lung cancer), AGS (stomach cancer), HT-29 (colon cancer) and HepG2 (liver cancer) were transfected simultaneously with an antisense macroarray composed of the 80 selected LC-antisense compounds.

[0273] Antisense compounds of the macroarray were mixed with various carriers such as peptides, DOTAP, and cationic liposomes in various ratios (w/w). The mixture was added to various amounts of cells according to their growth characteristics. Experimental cells treated with the LC-antisense compound-carrier complex and controls cells treated with carrier alone were incubated for 3 to 5 days and were subjected to MTT reduction assay twice. To examine the activity profile, the amount of growth inhibition was calculated, and the data were compared between the different types of cancerous cell lines (FIG. 19). Surprisingly, it was discovered that 7 LC-antisense compounds specifically, potently and differentially inhibited HepG2 cell growth (Table 2).

[0274] These results demonstrate that not only direct gene functionalization, but validation of target genes for molecular therapeutics to a particular disease can be performed simultaneously with the high-throughput system for functional genomics of the present invention.

[0275] All of the references cited herein are incorporated by reference in their entirety.

[0276] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention specifically described herein. Such equivalents are intended to be encompassed in the scope of the claims.

TABLE 1

Examples of Functionally Identified Genes Involved in the Growth of Liver Cancer Cells	
Name of the Gene	GenBank Accession Number
<i>Homo sapiens</i> , HSPC025, clone MGC:4223 IMAGE:2959747	BC007510
<i>Homo sapiens</i> , tissue inhibitor of metalloproteinase 1	XM_033878
<i>Homo sapiens</i> , alpha-fetoprotein (AFP)	NM_001134
<i>Homo sapiens</i> , hypothetical protein FLJ14075	NM_024894
<i>Homo sapiens</i> , apolipoprotein A-II (APOA2)	NM_001643
<i>Homo sapiens</i> , clone MGC:20176 IMAGE:3503710	BC018990
<i>Homo sapiens</i> , eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2)	NM_001967
<i>Homo sapiens</i> , cytochrome P450, subfamily IIE (ethanol-inducible)	XM_051310

TABLE 1-continued

Examples of Functionally Identified Genes Involved in the Growth of Liver Cancer Cells	
Name of the Gene	GenBank Accession Number
(CYP2E) <i>Homo sapiens</i> , Similar to serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1, clone MGC:9222 IMAGE:3859644	BC011991

[0277]

TABLE 2

Examples of Genes Which Specifically and Differentially Affect Cell Growth of HepG2	
Name of the Gene	GenBank Accession Number
EST_Human IL3-UT0117-160301-504-H11	BI062502
<i>Homo sapiens</i> , Apolipoprotein A-II, clone MGC:12334	BC005282
<i>Homo sapiens</i> , PRO2675 mRNA, complete cds	AF119890
<i>Homo sapiens</i> , clone RP11-449G13 from 16, complete sequence	AC020716
<i>Homo sapiens</i> , BAC clone RP11-360H4 from 2, complete sequence.	AC019086
<i>Homo sapiens</i> , hypothetical gene supported by AK023036 (LOC90271), mRNA	XM_030445
<i>Homo sapiens</i> , similar to cytochrome b5 outer mitochondrial membrane precursor (<i>H. sapiens</i>) (LOC124229), mRNA	XM_015216

[0278]

SEQUENCE LISTING

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tg 62

What is claimed is:

1. A library of a multitude of single-stranded large circular nucleic acids, said library comprising:

a multiplicity of compartments, each of said compartments comprising one or more single-stranded large circular antisense molecule of bacteriophage or phagemid vector comprising at least one unidirectional antisense nucleic acid insert,

wherein said large circular antisense molecule is capable of being introduced into a host cell, and is capable of specifically binding to a nucleic acid in said host cell that is substantially complementary to said antisense nucleic acid insert.

2. The library of claim 1, wherein the specificity of the antisense nucleic acid insert is unknown at the time said library is first made.

3. The library of claim 1, wherein said host cell is a eucaryotic cell.

4. The library of claim 1, wherein each of said compartments contains from about 0.1 μ M to about 1 μ M of said large circular antisense molecule.

5. The library of claim 1, wherein said bacteriophage or phagemid vector is derived from a filamentous bacteriophage.

6. The library of claim 5, wherein said filamentous bacteriophage is M13 bacteriophages.

7. The library of claim 1, wherein the source of said nucleic acid insert is an eucaryotic organism.

8. The library of claim 1, wherein said bacteriophage or phagemid vector comprises more than one kind of antisense nucleic acid insert sequence.

9. The library according to claim 1, wherein said multiplicity of compartments comprises a multiwell format of at least 6 wells.

10. The library according to claim 1, wherein said library is configured to be made and used in a substantially automated process.

11. The library according to claim 9, wherein said multiplicity of compartments comprises a multiwell format of at least 96 wells.

12. A method of making a library comprising a multitude of single-stranded large circular nucleic acids, which comprises one or more single-stranded bacteriophage or phagemid vector comprising at least one unidirectional antisense nucleic acid insert, comprising:

(i) inserting a nucleic acid fragment unidirectionally into said bacteriophage or phagemid vector by unidirectionally cloning the nucleic fragments into said vector;

(ii) preparing bacterial transformants by introducing the vector containing the insert into competent bacterial cells to make bacterial transformants; and

(iii) infecting said transformants with helper phage to produce said single-stranded nucleic acid library.

13. A library of a multitude of single-stranded large circular nucleic acids, said library comprising:

a multiplicity of compartments, each of said compartments comprising one or more single-stranded large circular antisense molecule of bacteriophage or phagemid vector comprising at least one unidirectional subtracted antisense nucleic acid insert,

wherein said large circular antisense molecule is capable of being introduced into a host cell, and is capable of specifically binding to a nucleic acid in said host cell that is substantially complementary to said antisense nucleic acid insert.

14. The library according to claim 13, wherein said unidirectional subtracted antisense nucleic acid is made by hybridizing a population of nucleic acids expressed from a first cell line or tissue with a population of nucleic acids expressed from a second cell line or tissue, and obtaining a nucleic acid population from the first cell line or tissue that does not hybridize with the nucleic acid population from said second cell line or tissue.

15. The library according to claim 14, wherein said first cell line or tissue is abnormal such that modulation of gene expression is beneficial in returning said first cell line or tissue to normal, and wherein said second cell line or tissue is normal.

16. The library according to claim 15, wherein said abnormality is cancer, viral infection, immunologic disorders or metabolic diseases.

17. The library according to claim 16, wherein said cancer is liver cancer, lung cancer, stomach cancer, colon cancer, leukemia, thyroid cancer, skin cancer, prostate cancer, cervical cancer, or breast cancer.

18. The library according to claim 16, wherein said viral infection is caused by human papilloma virus (HPV), HIV, small pox, mononucleosis (Epstein-Barr virus), hepatitis, or respiratory syncytial virus (RSV).

19. The library according to claim 16, wherein said metabolic disease is phenylketonuria (PKU), primary hypothyroidism, galactosemia, abnormal hemoglobins, types I and II diabetes, or obesity.

20. The library according to claim 16, wherein said immunological disorder is Sjogren's Syndrome, antiphospholipid syndrome, immune complex diseases, Purpura, Schoenlein-Henoch, immunologic deficiency syndromes, systemic lupus erythematosus, immunodeficiency, rheumatism, kidney, or liver sclerosis.

21. A method of making a library comprising a multitude of single-stranded large circular nucleic acids, which comprises one or more single-stranded bacteriophage or

phagemid vector comprising at least one unidirectional subtracted antisense nucleic acid insert, comprising:

- (i) inserting a subtracted nucleic acid fragment unidirectionally into said bacteriophage or phagemid vector by unidirectionally cloning the subtracted nucleic fragments into said vector;
- (ii) preparing bacterial transformants by introducing the vector containing the insert into competent bacterial cells to make bacterial transformants; and
- (iii) infecting said transformants with helper phage to produce said single-stranded nucleic acid library.

22. The method according to claim 21, wherein said subtracted nucleic fragment is made by hybridizing a population of nucleic acids expressed from a first cell line or tissue with a population of nucleic acids expressed from a second cell line or tissue, and obtaining a nucleic acid population from the first cell line or tissue that does not hybridize with the nucleic acid population from said second cell line or tissue.

23. A method for specifically inhibiting growth of liver cancer cells, comprising administering to said cells large circular antisense molecules targeted to EST_Human IL3-UT0117-160301-504-H11; Apolipoprotein A-II, clone MGC:12334; PRO2675 mRNA; clone RP11-449G13 from 16; BAC clone RP11-360H4 from 2; gene supported by AK023036 (LOC90271); or gene similar to cytochrome b5 outer mitochondrial membrane precursor (*H. sapiens*) (LOC124229).

24. A method for specifically inhibiting growth of liver cancer cells, comprising administering to said cells large circular antisense molecules targeted to HSPC025, clone MGC:4223 IMAGE:2959747; tissue inhibitor of metalloproteinase 1; alpha-fetoprotein (AFP); gene encoding protein FLJ14075; apolipoprotein A-II (APOA2); clone MGC:20176 IMAGE:3503710; eukaryotic translation initiation factor 4A, isoform 2 (EIF4A2); cytochrome P450, subfamily IIE (ethanol-inducible) (CYP2E); or gene similar to serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1, clone MGC:9222 IMAGE:3859644.

25. A high throughput system for functional genomics using a random gene unidirectional antisense library or random gene unidirectional subtracted antisense library comprising the following steps:

- (i) forming large circular antisense molecule-carrier complexes with said unidirectional or unidirectional subtracted antisense libraries;

- (ii) performing a primary gene functional analysis by transfecting the complexes into host cells to screen for the large circular antisense molecule that eliminates endogenously expressed substantially complementary transcripts;

- (iii) identifying the large circular antisense molecule that eliminates the endogenously expressed transcript; and

- (iv) sequencing either the antisense molecule or cDNA that corresponds to the antisense molecule.

26. The high throughput system according to claim 25, further comprising,

- (v) performing further gene function analysis with the large circular antisense molecule identified in steps (iii) and (iv).

27. The high throughput system according to claim 25, comprising comparing the gene sequence obtained in step (iv) with a DNA sequence database to identify the gene.

28. The high throughput system according to claim 25, wherein the carrier is liposomes, cationic polymers, HIV-liposomes complexes, peptides or viruses.

29. The high throughput system according to claim 25, wherein the large circular antisense molecule and carrier are mixed in an optimal ratio of about 1:3 to about 1:4 by weight.

30. The high throughput system according to claim 26, wherein the gene function analysis is assaying for the phenotype of cell morphology, cell proliferation, cell apoptosis, or cell reaction to a substrate.

31. The high throughput system according to claim 26, wherein said gene function analysis is carried out by performing an assay, wherein said assay is RT-PCR, Western blot analysis, immunoassay, MTT reduction assay, [³H]-thymidine incorporation assay, colony formation assay, DNA synthesis and chromatin activation, analysis of apoptosis by inspection of cell morphological changes, chromosomal condensation or fragmentation, DNA fragmentation, quantitative assay for apoptosis, signaling mechanisms of apoptosis, activation of cell cycle regulators, complex formation between cell cycle regulators, or assays for changes of metabolic, morphological, physiological and biochemical phenotypes in vitro and in vivo.

* * * * *