



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US98/13043 <b>(22) International Filing Date:</b> 24 June 1998 (24.06.98)  <b>(30) Priority Data:</b> 60/050,729              25 June 1997 (25.06.97)              US  <b>(71) Applicant:</b> LIFE TECHNOLOGIES, INC. [US/US]; 9800 Medical Center Drive, Rockville, MD 20850 (US).  <b>(72) Inventors:</b> LI, Wu-bo; 11909 Riding Loop Terrace, North Potomac, MD 20878 (US). JESSE, Joel; 4139 Old National Pike, Mt. Airy, MD 21771 (US). NISSON, Paul; 9504 Appeals Place, Gaithersburg, MD 20879 (US).  <b>(74) Agents:</b> AUERBACH, Jeffrey, I. et al.; Howrey & Simon, 1299 Pennsylvania Avenue, N.W., Box 34, Washington, DC 20004-2402 (US).	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> IMPROVED METHOD FOR ISOLATING AND RECOVERING TARGET DNA OR RNA MOLECULES HAVING A DESIRED NUCLEOTIDE SEQUENCE		
<b>(57) Abstract</b>		
<p>The invention generally concerns the use of amino acid denaturants for denaturing or separating double stranded nucleic acid molecules. More specifically, the present invention provides a method for the rapid isolation and recovery of a desired target DNA or RNA molecules from a mixture or library containing such molecules. The method involves the use of haptentylated probes and amino acid denaturants to select the desired molecules and eliminate the undesired library members from a sample. The invention also provides a method in which larger or full-length nucleic acid molecules can be isolated from the subpopulation of desired molecules.</p>		

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**TITLE OF THE INVENTION:****Improved Method For Isolating And Recovering Target DNA Or  
RNA Molecules Having A Desired Nucleotide Sequence****FIELD OF THE INVENTION:**

5           The invention relates to an improved method for isolating and recovering target DNA or RNA molecules having a desired nucleotide sequence. Specifically, it relates to a method for the rapid isolation of specific nucleic acid target molecules.

**BACKGROUND OF THE INVENTION:**

10           The ability to clone gene sequences has permitted inquiries into the structure and function of nucleic acids, and has resulted in an ability to express highly desired proteins, such as hormones, enzymes, receptors, antibodies, etc., in diverse hosts.

15           The most commonly used methods for cloning a gene sequence involve the *in vitro* use of site-specific restriction endonucleases, and ligases. In brief, these methods rely upon the capacity of the "restriction endonucleases" to cleave double-stranded DNA in a manner that produces termini whose structure (i.e., 3' overhang, 5' overhang, or blunt end) and sequence are both well defined. Any such DNA molecule can then be  
20           joined to a suitably cleaved vector molecule (i.e., a nucleic acid molecule, typically double-stranded DNA, having specialized sequences which permit it to be replicated in a suitable host cell) through the action of a DNA ligase. The gene sequence may then be duplicated indefinitely by propagating the vector in a suitable host. Methods for performing such manipulations are  
25           well-known (see, for example, Perbal, B. *A Practical Guide to Molecular Cloning*, John Wiley & Sons, NY, (1984), pp. 208-216; Sambrook, J., *et al.* (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982); Old, R.W. *et al.*, In: *Principles of Gene*

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*Manipulation*, 2nd Ed., University of California Press, Los Angeles, (1981), all herein incorporated by reference).

5 In some cases, a gene sequence of interest is so abundant in a source that it can be cloned directly without prior purification or enrichment. In most cases, however, the relative abundance of a desired target DNA molecule will require the use of ancillary screening techniques in order to identify the desired molecule and isolate it from other molecules of the source material.

10 A primary screening technique involves identifying the desired clone based upon its DNA sequence via hybridization with a complementary nucleic acid probe.

15 *In situ* filter hybridization methods are particularly well known (see, Sambrook, J., *et al.*, In: *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). In such methods, bacteria are lysed on the surface of the membrane filter and then incubated in the presence of a detectably labeled nucleic acid molecule whose sequence is complementary to that of the desired sequence. If the lysate contains the desired sequence, hybridization occurs and thereby binds the labeled molecule to the adsorbent surface. The detection of the label on the adsorbent surface reveals that the bacteria sampled contained the desired cloned sequence.

20 Although these screening methods are useful and reliable, they require labor-intensive and time consuming steps such as filter preparation and multiple rounds of filter hybridization and colony platings/phage infections. Generally, these procedures will screen up to  $10^6$  colonies effectively, but may take weeks or months to yield the desired clone.

25 Other approaches have been developed to isolate recombinant molecules which have eliminated the tedious filter-handling procedure. These approaches employ conventional hybridization technology coupled with chromatography or magnetic particle technology. Rigas, B. *et al.*, for example, reported a method for isolating one plasmid species from a mixture of two plasmid species. In the disclosed method, circular double-stranded plasmid DNA is hybridized to a RecA protein-coated biotinylated

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probe to form a stable triple-stranded complex, which is then selectively bound to an agarose-streptavidin column (Rigas, B. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 83: 9591-9595 (1986)). The method thus permits the isolation of cloned double-stranded molecules without requiring any separation of the strands.

5 A DNA isolation method, termed "triplex affinity capture," has been described in which a specific double-stranded genomic DNA is hybridized to a biotinylated homopyrimidine oligonucleotide probe to form a "triplex complex," which can then be selectively bound to streptavidin-coated magnetic beads (Ito, T. *et al.*, *Nucleic Acids Res.* 20: 3524 (1992); Ito, T. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 89: 495-498 (1992)). Takabatake, T. *et al.* have described a variation of this technique that employs a biotinylated purine-rich oligonucleotide probe to detect and recover the desired nucleic acid molecule (Takabatake, T. *et al.*, *Nucleic Acid Res.* 20: 5853-5854 (1992)). A practical drawback with these particular approaches is that they are restricted to isolation of target DNA sequences containing homopurine-homopyrimidine tracts.

10 Fry, G. *et al.* discuss a method for sequencing isolated M13-*LacZ* phagemids (Fry, G. *et al.*, *BioTechniques* 13:124-131 (1992)). In this method, a clone is selected and the phagemid DNA is permitted to hybridize to a biotinylated probe whose sequence is complementary to the phagemid's *lacZ* region. The biotinylated probe is attached to a streptavidin-coated paramagnetic bead. Since the DNA bound to the bead can be separated from unbound DNA, the method provides a means for separating the cloned sequence from the bacterial sequences that are inevitably present (Fry, G. *et al.*, *BioTechniques* 13: 124-131 (1992)).

20 Still another method of screening recombinant nucleic acid molecules is described by Kwok, P.Y. *et al.* This method, which is an extension of PCR-based screening procedures uses an ELISA-based oligonucleotide-ligation assay (OLA) to detect the PCR products that contain the target source (Kwok, P.Y. *et al.*, *Genomics* 13: 935-941 (1992)). The OLA employs an "reporter probe" and a phosphorylated/biotinylated

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"anchor" probe, which is captured with immobilized streptavidin (Landegren, U. *et al.*, *Science* 241:1077-1080 (1988)).

The isolation of target DNA from a complex population using a subtractive hybridization technique has also been described (Lamar, E.E. *et al.*, *Cell* 37:171-177 (1984); Rubenstein, J.L.R. *et al.*, *Nucleic Acids Res.* 18:4833-4842 (1990); Hedrik, S.M. *et al.*, *Science* 308:149-153 (1984); Duguid, J.R. *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 85:5738-5742 (1988)). In such "subtractive hybridization" screening methods, the cDNA molecules created from a first population of cells is hybridized to cDNA or RNA of a second population of cells in order to "subtract out" those cDNA molecules that are complementary to nucleic acid molecules present in the second population and thus reflect nucleic acid molecules present in both populations.

The method is illustrated by Duguid, J.R. *et al.* (*Proc. Natl. Acad. Sci. (U.S.A.)* 85:5738-5742 (1988)) who used subtractive hybridization to identify gene sequences that were expressed in brain tissue as a result of scrapie infection. A cDNA preparation made from uninfected cells was biotinylated and permitted to hybridize with cDNA made from infected cells. Sequences in common to both cDNA preparations hybridized to one another, and were removed from the sample through the use of a biotin-binding (avidin) resin.

Weiland, I. *et al.* (*Proc. Natl. Acad. Sci. (U.S.A.)* 87:2720-2724 (1990)) reported an improved method of subtractive hybridization in which tester DNA was cleaved with a restriction endonuclease, and then permitted to hybridize to sheared driver DNA at high  $C_0t$  values (" $C_0t$ " is the product of the initial concentration of DNA and the time of incubation). By cloning the double-stranded, PCR-amplified, unique DNA molecules into a plasmid vector, it was possible to obtain an enrichment in the relative proportion of target sequences recovered.

Rubenstein, J.L.R. *et al.* (*Nucleic Acids Res.* 18:4833-4842 (1990)) reported a further improvement in subtractive hybridization methods that employed single-stranded phagemid vectors to provide both the target and tester DNA. In the method, hybridized phagemid DNA-biotinylated driver

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strand complexes are separated from unhybridized DNA by the addition of streptavidin. Unhybridized single-stranded DNA was subsequently converted to the double-stranded form using Taq DNA polymerase and an oligonucleotide complementary to a common region found within the single-stranded DNA. The use of this method is, however, limited by the need to follow a rigorous single-stranded phagemid purification protocol in order to obtain a preparation virtually free of contaminant double-stranded DNA (Rubenstein, J.L.R. *et al.*, *Nucleic Acids Res.* 18: 4833-4841 (1990)).

In sum, methods for isolating particular target nucleic acid molecules are restricted by the abundance of the DNA target sequence, and by time-consuming steps. Accordingly, a method that would expedite the isolation of desired target nucleic acid molecules and that could yield essentially pure target DNA would be highly desirable.

#### SUMMARY OF THE INVENTION:

The present invention provides a method for rapidly isolating nucleic acid molecules having a desired nucleotide sequence from other undesired nucleic acid molecules. In particular, the invention allows for isolation of a desired nucleic acid molecule from a population of nucleic acid molecules. Significantly, the present invention further relates to an improved method of screening target nucleic acid molecules employing hybridization methodology combined with ligand separation, DNA repair, and restriction enzyme digestion technology.

In detail, the invention provides a method for selectively isolating a desired target nucleic acid molecule present in an initial sample containing a mixture (or library) of nucleic acid molecules, wherein said method comprises the steps:

- (a) (1) where said initial mixture or library is composed of single-stranded nucleic acid molecules, performing step (b); or
- (2) where said initial mixture or library is composed of double-stranded nucleic acid molecules treating said double-stranded nucleic acid molecules to render such molecules single-stranded, then performing step (b);

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- 5 (b) incubating single-stranded nucleic acid molecules of said mixture or library in the presence of haptenylated nucleic acid probe molecules, said probe molecules comprising a nucleotide sequence complementary to a nucleotide sequence of said desired target molecule; said incubation being under conditions sufficient to permit said probe molecules to hybridize to said desired target molecules, thereby generating hybridized molecules wherein said desired target molecules are bound to said probe molecules;
- 10 (c) capturing said hybridized molecules of step (b) by incubating said hybridized molecules in the presence of a binding ligand of the hapten of said haptenylated probes, said binding ligand being conjugated to a support; said incubation being sufficient to permit said hybridized molecules to become bound to said binding ligand of said support;
- 15 (d) separating said bound hybridized desired target molecules from unbound nucleic acid molecules; and
- (e) recovering said desired target molecules from said support.

20 In a preferred embodiment, the invention concerns the use of one or more amino acid denaturants for separating double-stranded nucleic acid molecules. Such amino acid denaturants allow separation of complementary strands of double stranded nucleic acid molecules formed by hybridization. In particular these amino acid denaturants provide separation of the double-stranded nucleic acid molecule mixture prior to hybridization with the haptenylated nucleic acid probes (step (a)) and preferably are used to separate the probes from the desired nucleic acid molecule. In a particularly preferred embodiment of the invention, the desired nucleic acid molecules are recovered by incubating the support containing the bound probes hybridized to the desired molecules with one

25 or more amino acid denaturants. Such incubation is carried out under conditions sufficient to release the desired molecules from the probes.

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According to the invention, an amino acid denaturant includes any amino acid, polyamino acid or derivative thereof which can be used to

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dissociate or denature double stranded nucleic acid molecules. Such amino acid denaturants include, but are not limited to, glycine, alanine, arginine, asparagine, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and imidazole.

5 Thus, the method of the present invention is more particularly directed to recovering one or more desired target nucleic acid molecules from a sample comprising:

(a) contacting said sample in the presence of one or more haptenylated nucleic acid probes comprising a nucleotide  
10 sequence complementary to said desired target molecules under conditions sufficient to permit said probes to hybridize to said desired target molecules thereby forming one or more hybridized molecules;

(b) contacting said hybridized molecules with binding ligands  
15 conjugated to a support under conditions sufficient to permit said hybridized molecules to become bound to said binding ligands of said support; and

(c) contacting said support with one or more amino acid  
20 denaturants under conditions sufficient to isolate said desired nucleic acid molecules from said support.

This method of the invention may further comprise:

(d) contacting said isolated desired nucleic acid target molecules  
25 with one or more primers complementary to one or more sequences of the desired target nucleic acid molecules under conditions sufficient to generate one or more double-stranded desired nucleic acid target molecules; and

(e) transforming said double-stranded desired target molecules  
into one or more host cells.

30 In this aspect of the invention, the double-stranded desired target molecule may be produced by incubating the desired target molecules with one or more primers, one or more nucleotides, and a polypeptide having polymerase activity. Such polypeptides having polymerase activity include well known DNA and/or RNA polymerases, preferably thermostable DNA

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polymerases. Nucleotides for use in this embodiment include but are not limited to dATP, dGTP, dCTP, dTTP, ATP, GTP, CTP, UTP, and analogs thereof. In particular, nucleotide analogs that confer nuclease or endonuclease resistance to the synthesized nucleic acid molecule are particularly preferred. When such nucleotide analogs are used in accordance with the invention, the methods of the invention may further comprise incubating the double-stranded desired target molecule (which contains one or more nucleotide analogs) with one or more nucleases or endonucleases prior to transformation. Incubating such molecules in this manner provides for an additional selection step against contaminating nucleic acid molecules which do not contain such nucleotide analogs. The present invention also concerns the use of unique primers which recognize and hybridize to the desired target nucleic acid molecules. Such primers include sequences which are complementary to the same sequence recognized by the probe molecule or may be complementary to a different sequence within the target nucleic acid molecules. In particularly preferred embodiments, the probes and/or primers are degenerate oligonucleotides, preferably degenerate oligonucleotides which contain one or more universal nucleotides.

The present invention also relates to a method for selecting or enriching for desired target nucleic acid molecules having larger or longer segments from a population of desired target nucleic acid molecules. As will be appreciated, selection of desired nucleic acid molecules in accordance with the invention provides a population of desired molecules which hybridize to the probe. In such a population, the length or size of the sequence contained in each target nucleic acid molecule may vary. In the enrichment method of the invention, the desired nucleic acid molecules having larger segments or larger sequences can be selected by separating the desired nucleic acid molecules according to size. Such size separation can be accomplished by well known techniques including gel electrophoresis (e.g., agarose or acrylamide). Upon separation, larger nucleic acid molecules can be isolated and then utilized for further processing.

In a particular preferred aspect, the enrichment procedure is used to screen cDNA molecules contained in a vector. In such a procedure, the cDNA molecules prepared from messenger RNA or polyA+ RNA are cloned into a vector, thereby forming a cDNA library. Given that the vector is a constant size, selection of larger molecules from the library provides for vectors containing the largest cDNA inserts. In this manner, larger or full length cDNA molecules may be isolated from the cDNA library. This aspect of the invention thus provides a means to select full length desired genes from a cDNA library. In a preferred enrichment method of the invention, the desired target molecules within the cDNA library are amplified prior to size separation.

Thus the invention specifically relates to enrichment of desired nucleic acid molecules having larger or full length inserts comprising:

- (a) obtaining a cDNA library;
- (b) (1) where said library is composed of single-stranded nucleic acid molecules, performing step (c); or (2) where said library is composed of double-stranded nucleic acid molecules treating said double-stranded nucleic acid molecules to render such molecules single-stranded, then performing step (c);
- (c) contacting single-stranded nucleic acid of said library with one or more haptenylated nucleic acid probes comprising a nucleotide sequence complementary to a nucleotide sequence of one or more desired target molecules;
- (d) isolating said desired target molecules;
- (e) amplifying said isolated desired target molecules; and
- (f) separating said amplified molecules according to size.

Of course, the enrichment method of the invention may be used on any nucleic acid populations, not only cDNA libraries. In such a method, the population of nucleic acid molecules (preferably contained in a vector) are used to select a subpopulation of desired target nucleic acid molecules. The subpopulation of desired nucleic acid molecules (each molecule likely having a different size) are then separated according to size, preferably after amplification. In the enrichment methods of the invention, regardless of

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the sample used (cDNA library or other nucleic acid populations), the type and number of probes used for amplification may vary.

#### BRIEF DESCRIPTION OF THE FIGURES:

5 Figure 1 provides a diagrammatic illustration of a preferred embodiment of the isolation method of the present invention.

Figure 2 provides a diagrammatic view of a preferred method for generating single-stranded nucleic acid molecules.

Figure 3 provides a diagrammatic view of a preferred method for performing PCR on an enriched population of molecules.

#### 10 DESCRIPTION OF THE PREFERRED EMBODIMENTS:

The present invention concerns an improved method for rapidly isolating a "desired" nucleic acid "clone" from a mixture (or library of cloned molecules). The "clones" of the present invention comprise circular or linear DNA or RNA molecules that may be either single-  
15 stranded or double-stranded. Typically, such clones or libraries will comprise plasmids or other vectors (such as viral vectors) that have been engineered to contain a "desired" fragment of DNA or RNA derived from a source such as a homogeneous specimen (such as cells in tissue culture, cells of the same tissue, etc.), or a heterogeneous specimen (such as a  
20 mixture of pathogen-free and pathogen-infected cells, a mixture of cells of different tissues, species, or cells of the same or different tissue at different temporal or developmental stages, etc.). The cells, if any, of these nucleic acid sources may be either prokaryotic or eukaryotic cells (such as those of animals, humans and higher plants).

25 Various libraries can be selected for large scale preparation. The construction of plasmid, cosmid, and phagemid cDNA libraries, or genomic libraries are described in Sambrook, J. et al. (In: Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), herein incorporated by reference).  
30 Preferably, single-stranded phagemid cDNA libraries can be prepared as described previously by Gruber, C.E. *et al.*, (*Focus* 15:59-65 (1993), herein incorporated by reference). The general steps of the method will differ

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depending upon whether the desired sequence has been cloned into single-stranded or double-stranded molecules, and whether such molecules are DNA or RNA.

5 As used herein, there is no constraint as to the sequence of the target nucleic acid molecule whose isolation is desired. Since the present invention relies upon nucleic acid hybridization, the target molecules should have a length of at least 10 nucleotides in order to be efficiently recovered. No upper limit to the size of the molecules exists, and the methods of the invention can be used to isolate nucleic acid molecules of  
10 several kilobases or more.

The selection method of the present invention is based in part upon the observation that double-stranded nucleic acid molecules transform bacterial cells with greater efficiency than single-stranded nucleic acid molecules. In one embodiment, the invention achieves the isolation of a  
15 desired nucleic acid sequence from a library of sequences by providing a primer molecule to the mixture. A "primer" or "primer molecule" as used herein is a single-stranded oligonucleotide or a single-stranded polynucleotide that can be extended by the covalent addition of nucleotide monomers during the template-dependent polymerization reaction  
20 catalyzed by a polymerase. A primer is typically 11 bases or longer; most preferably, a primer is 17 bases or longer. However, the primer may range in size from 16 to 300 bases, preferably 16 to 32 bases and most preferably 20 to 24 bases. Examples of suitable DNA polymerases include the large proteolytic fragment of the DNA polymerase I of the bacterium *E. coli*,  
25 commonly known as "Klenow" polymerase, *E. coli* DNA polymerase I, the bacteriophage T7 DNA polymerase. Preferably, a thermostable polymerase will be used, such as a polymerase that can catalyze nucleotide addition at temperatures of between about 50°C to about 100°C. Additionally, combinations of polymerases may be used to increase the efficiency of  
30 polymerization, such as Elongase (Life Technologies, Inc., Gaithersburg, Maryland). Exemplary thermostable polymerases are described in European Patent Application No. 0258017, incorporated herein by reference. The thermostable "Taq" DNA polymerase (Life Technologies,

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Inc., Gaithersburg, Maryland) is an example, although other well known thermostable polymerases and their mutants and derivatives thereof may be used, such as Tne DNA polymerase (WO96/10640, copending Application Serial No. 08/706,706, filed September 6, 1996 and copending  
5 application 60/037,393, filed February 7, 1997), Tma DNA polymerase (U.S. Patent No. 5,374,553), Pfu DNA polymerase (U.S. Patent No. 5,489,523), Vent DNA polymerase (U.S. Patent Nos. 5,210,036, 5,500,363, 5,352,778, and 5,322,785), DEEPVENT® (New England Biolabs), Dynazyme (Finnzymes, Finland), and Tfl (Epicenter Technologies, Inc.).

10 Where the target mixture involved RNA molecules, and a DNA molecule is desired, a reverse transcriptase may be employed. Reverse transcriptases are discussed by Sambrook, J. *et al.* (In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)) and by Noonan, K. F. *et al.* (*Nucleic Acids Res.* 16:10366  
15 (1988)). Preferably, reverse transcriptases substantially lacking RNase H activity (U.S. Patent No. 5,244,797) are used. Such reverse transcriptases may be obtained from Life Technologies, Inc. (Gaithersburg, Maryland). Similarly, where the target mixture comprises RNA, an RNA polymerase may be used. Examples of suitable RNA polymerases include *E. coli* RNA  
20 polymerase, T7 RNA polymerase, etc.

As a consequence of such polymerization, the desired target molecules, but not other nucleic acid molecules of the mixture, are converted into a double-stranded form. The mixture can, without further processing, be transformed into suitable recipient bacteria (see, Sambrook, J.  
25 *et al.*, In: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). Transformants can be recovered, and their recombinant DNA or RNA molecules can be extracted and retrieved. Such processing provides a new mixture or library of nucleic acid molecules that is substantially enriched for the desired  
30 molecules. Optionally, the above-described method can be repeated (as often as desired) in order to obtain mixtures or libraries that are more highly enriched for the desired nucleic acid sequence.

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A preferred method for conducting such processing employs a library or mixture of a single-stranded phagemid, such as M13, or from vectors such as pSPORT 1, pCMV•SPORT (particularly DNA cloned into the Not I-Sal I region), pZL1 ( $\lambda$ Ziplox<sup>®</sup>), P1, PAC, YAC, BAC and BlueScript SK (+). In a preferred embodiment of the method, a primer is used to convert the single-stranded DNA molecule into a double-stranded form. When using a single-stranded phagemid vector, care must be taken to select an oligonucleotide with the correct polarity. If the target gene is cloned into multiple cloning sites in the same orientation as the lacZ gene, sense strand (i.e., the strand containing the ATG initiation codon for protein synthesis) sense oligonucleotides need to be used to capture ssDNA produced from vectors such as pSPORT 1, pCMV•SPORT (particularly DNA cloned into the Not I-Sal I region), pZL1 (and BlueScript SK (+). Anti-sense (non-ATG stand) oligonucleotides are used to capture ssDNA produced from vectors such as pSPORT2, BlueScript SK (-), and  $\lambda$ Zap<sup>®</sup>II. If ssDNA is generated by in vivo phagemid production, oligonucleotide of the reverse polarity must be designed (i.e., anti-sense oligonucleotides for pSPORT 1, pCMV•SPORT, etc.).

A highly preferred embodiment of the present invention is marketed by Gibco BRL (GeneTrapper<sup>™</sup> cDNA Positive Selection System, Life Technologies, Inc. (Gaithersburg, Maryland), the instruction manual of which is herein incorporated by reference in its entirety). Also incorporated by reference in its entirety is U.S. Patent No. 5,500,356 to Li *et al.* regarding a method of nucleic acid sequence selection. This embodiment of the present invention facilitates the rapid (1 to 2 days) isolation of cDNA clones from DNA prepared from a cDNA library (representing, for example,  $10^{12}$  DNA molecules) with no prior cDNA library screening. In this system (Figure 1), an oligonucleotide, complementary to a segment of the target cDNA, is biotinylated at the 3' end with biotin-14-dCTP using terminal deoxynucleotidyl transferase ("TdT"). Simultaneously, a complex population of double-stranded phagemid DNA containing cDNA inserts (e.g.,  $10^6$  to  $10^7$  individual members) is converted to single-stranded DNA ("ssDNA") using Gene II

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(phage F1 endonuclease) and (*E. coli*) Exonuclease III (Exo III). Hybrids between the biotinylated oligonucleotide and ssDNA are formed in solution and are then captured on streptavidin-coated paramagnetic beads. A magnet is used to retrieve the paramagnetic beads from solution, leaving nonhybridized ssDNA behind in solution. Subsequently, the captured ssDNA target is released from the biotinylated oligonucleotide that remains attached to the paramagnetic beads. After release, the desired cDNA clone is further enriched by using a non-biotinylated target oligonucleotide to specifically prime conversion of the recovered ssDNA target to double stranded DNA ("dsDNA"). The term "repair" as used herein refers to the conversion of ssDNA into dsDNA. Following transformation and plating, typically, 20% to 100% of the colonies represent the cDNA clone of interest. If the percent representation of the target cDNA species is unknown, the repair step is preferably used to ensure adequate enrichment of the target cDNA.

The GeneTrapper™ System provides several distinct advantages over PCR (GeneTrapper™ cDNA Positive Selection System, Life Technologies, Catalog No. 10356-020, herein incorporated by reference in its entirety). Cloned, full-length cDNAs can be easily isolated by using the GeneTrapper™ System and one specific oligonucleotide of  $\geq 16$  nucleotides that is designed to anneal to the 5' coding region. To obtain the same result from PCR would require sequence information at the 5' and 3' regions of the desired cDNA (two oligonucleotides) or a more difficult combined 3' – 5' procedure followed by a cloning procedure.

Oligonucleotide probes designed to the sequence information as close to the 5'-terminus of the target nucleic acid molecule will tend to enrich for full-length cDNA clones. On the other hand, oligonucleotides containing sequences proximal to the 3'-terminus of the original mRNA will select partial, full-length, and all other related cDNA clones (i.e., spliced transcripts).

In accordance with the invention, the GeneTrapper system may be modified by one or a combination of improvements including (1) utilizing degenerate oligonucleotides (particularly oligonucleotides containing

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universal nucleotides such as dP and/or dK) as primers and/or as haptenylated probes, (2) utilizing one or more amino acid denaturants to convert the double-stranded nucleic acid molecules into single-stranded nucleic acid molecules, (3) utilizing nucleotide analogs during the repair  
5 reaction to confer nuclease resistance; and (4) enrichment of larger or full-length nucleic acid molecules.

#### A. Capture Enrichment of Desired Molecules

The selection method of the present invention employs a nucleic acid "capture" step. This embodiment is preferably performed using single-  
10 stranded nucleic acid molecules. Where double-stranded circular molecules are employed, a preferred initial step involves denaturing (or otherwise separating) the molecules into their respective single strands. Such denaturation may be accomplished by transient incubation of the sample at elevated temperatures (60-80°C or above the  $T_m$  of the mixture),  
15 or preferably by the use of one or more amino acid denaturants. Alternatively, salt or ionic conditions can be adjusted, or denaturation can be accomplished via helicase activity. The strand-separation step may require a topoisomerase in order to permit full strand separation. Alternatively, the double-stranded plasmid or linear target DNA could be  
20 nicked and the nicked strand removed by denaturation or digestion.

A preferred method for accomplishing such nicking and strand removal involves employing double-stranded circular molecules that contain a region of an origin of replication of an isometric or filamentous bacteriophage. Isometric bacteriophage include  $\phi$ X174, G4, G13, S13, St-1,  
25  $\phi$ K, U3, G14,  $\alpha$ 3 and G6. Filamentous bacteriophage include f1, fd, M13, If1, and Ike. Origin regions of M13 and fd are preferred (Baas, P.D. *et al.*, *Curr. Top. Microbiol. Immunol.* 136:31-70 (1988); Baas, P.D., *Biochim. Biophys. Acta* 825:111-139 (1985), both herein incorporated by reference).

Various bacteriophage proteins, and in particular, the Gene II  
30 protein of fd, and its analogs, can cleave a specific site in the region of an origin of replication of an isometric bacteriophage. Thus, by incubating such proteins with a double-stranded circular molecule that contains an isometric bacteriophage origin of replication region, it is possible to nick

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one strand of the circular molecule (Meyer, T.F. *et al.*, *Nature* 278:365-367 (1979) herein incorporated by reference). By further incubating the nicked molecule in the presence of an exonuclease (such as Exonuclease III), it is possible to degrade the nicked strand and obtain a preparation of circular single-stranded molecules (Chang, D.W. *et al.*, *Gene* 127:95-98 (1993); Eastlake, P.B. *et al.*, PCT Application No. WO95/09915, both herein incorporated by reference). Gene II - Exo III prepared ssDNA is in the opposite polarity to ssDNA generated by *in vivo* phagemid production.

In another aspect of the invention, the double-stranded molecules (preferably double-stranded circular molecules) are denatured by contacting the double-stranded molecules with one or more amino acid denaturants. Such amino acid denaturants includes any amino acid, polyamino acid, or derivative thereof which can be used to dissociate or denature double-stranded nucleic acid molecules. Such amino acids comprise one or more amino acids selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, imidazole, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and derivatives or analogs thereof; although, glycine, alanine, arginine, asparagine, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, imidazole, and derivatives or analogs thereof are preferred. Polyamino acids comprise two or more of such amino acids as well as their derivatives or analogs thereof. In accordance with the invention, any number of amino acids (and derivatives or analogs thereof) may be combined with any number of polyamino acids (and derivatives or analogs thereof) to denature double-stranded nucleic acid molecules. In the method of the invention, the amino acid denaturants allow for separation or denaturation of the double-stranded nucleic acid molecules to form single-stranded molecules. Contrary to the strand removal method (above), amino acid denaturants produce single-stranded molecules representing both strands of the double-stranded nucleic acid molecules. Preferably, amino acid denaturants are provided in a solution or as a buffer. The concentration of the amino acid

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denaturants in such buffers or solutions which is sufficient to denature or dissociate the double-stranded DNA molecule may be easily determined by one of ordinary skill in the art, taking in the consideration the amount and size of the double-stranded molecules. Typically, amino acid denaturants are used at a concentration from 1-500 mM, preferably 1-100 mM, more preferably 1-50 mM, still more preferably 5-50 mM, and most preferably 10-30 mM.

In accordance with the present invention, the population of single-stranded molecules is then incubated in the presence of one or more oligonucleotide probes under conditions sufficient to permit and promote sequence-specific nucleic acid hybridization. Hybridization may be conducted under conditions which either permit or minimize random hybridization. As used herein, conditions which minimize random hybridization are of such stringency that they permit hybridization only of sequences that exhibit complete complementarity. In contrast, conditions that permit random hybridization will enable molecules having only partial complementarity to stably hybridize with one another. Suitable conditions which either permit or minimize random nucleic acid hybridization are described by Sambrook, J., et al. (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982)); Haymes, B.D., et al. (In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), both herein incorporated by reference), and similar texts.

The probe is a nucleic acid molecule, preferably DNA, preferably greater than 8-12 nucleotides in length, and most preferably greater than 15-30 nucleotides in length, whose sequence is selected to be complementary to the sequence of a region of the target molecule that is to be isolated. However, the probe may range from 16 to 300 bases, preferably 16-32 bases and most preferably 20-24 bases. The probe thus need not be, and most preferably will not be equal in size to the target molecule that is to be recovered. The oligonucleotide probe will preferably have G+C content of from about 50% to about 60%. A higher G+C content will increase the number of background colonies.

Two sequences are said to be "complementary" to one another if they are capable of hybridizing to one another to form a stable anti-parallel, double-stranded nucleic acid structure. Thus, the sequences need not exhibit precise complementarity, but need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure. Thus, departures from complete complementarity are permissible, so long as such departures are not sufficient to completely preclude hybridization to form a double-stranded structure. However, complementarity determines the specificity of the capture reaction.

In one embodiment, the probe (and/or primer) may contain nucleotide analogues that are capable of hybridizing to more than one species of the four naturally occurring deoxynucleotides (dC, dG, dT, and dA). 2'-deoxyInosine or 2'-deoxyNebularine which exhibit low, but unequal, hydrogen bonding to the all four bases may be employed for such purpose. Alternatively, a "universal nucleotide" may be employed. In this strategy, the base analog does not hybridize significantly to any of the four bases. 3-Nitropyrrole 2'-deoxynucleoside, and 5-nitro-indole are examples of such a universal bases (Nichols, R. *et al.*, *Nature* 369:492-493 (1994); Loakes, D. *et al.*, *Nucl. Acids Res.* 22:4039-4043 (1994)). Nucleotides having bases capable of hybridizing to multiple species of nucleotides, as well as "universal nucleosides" may be obtained from Glen Research (Lin *et al.*, *Nucleic Acids Res.* 17:10373-10383 (1989); and Line *et al.*, *Nucleic Acids Res.* 20:5149-5152 (1992)). Examples of such universal nucleotides include dP and dK, obtainable from Glen Research.

Additionally, the probes (and/or primers) used in accordance with the invention may be protein nucleic acids (PNA's) (U.S. Patent No. 5,539,082, herein incorporated by reference). Use of such protein nucleic acids may allow for increased strength of binding of the probe (and/or primer) to the nucleic acid molecule.

In another embodiment, the sequence of the probe (and/or the primer) may be derived from amino acid sequence data. In these instances, the probe (and/or the primer) may have a degenerate sequence. For instance, if one had an amino acid motif (e.g., zinc fingers) that occurred in

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a number of proteins encoded in a library, one could enrich for nucleic acids encoding proteins having that motif. By designing the oligonucleotide probe to the amino acid encoding region of the cDNA, the capture of vector sequences will be avoided.

5 In a preferred sub-embodiment, the probe is "haptenylated." As used herein, a "haptenylated" probe is a nucleic acid molecule that has been covalently bonded to one or more of the same or different hapten molecules. A hapten is a molecule that can be recognized and bound by another molecule, e.g., a ligand. Examples of haptens include any antigen,  
10 biotin, dinitrophenol, etc. Biotin is a preferred hapten of the present invention and may be bound by proteins such as avidin and streptavidin.

The probe may be "haptenylated" using any of a variety of methods well known in the art. Methods for "biotinylation" the probe are described, for example, by Hevey et al. (U.S. Patent No. 4,228,237); Kourilsky et al.  
15 (U.S. Patent No. 4,581,333); Hofman *et al.* (*J. Amer. Chem. Soc.* 100:3585-3590 (1978)); Holmstrom, K. *et al.* (*Anal. Biochem.* 209:278-283 (1993)); etc. Such modification is most preferably accomplished by incorporating biotinylated nucleotides into a nucleic acid molecule using conventional methods. Alternatively, such modification can be made using photobiotin  
20 (Vector Laboratories). Other methods can, of course, be employed to produce such biotinylated molecules.

The formation of dimers or hairpin structure at the 3' terminus of the oligonucleotide probe will reduce or eliminate the ability of TdT to add biotin to the oligonucleotide. To avoid hairpin formation, oligonucleotide  
25 programs such as OLIGO™ 4.0 or OLIGO™ 5.0 for Windows may be used to design the oligonucleotide probe.

In a highly preferred method, a single biotinylated nucleotide species is employed (e.g., biotinylated dCTP), and the nucleotide is incorporated into the probe molecule either throughout the length of the probe, or,  
30 more preferably, at an end of the probe, such that a homopolymeric region is created (e.g., poly-biotinylated dC).

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The above-described incubation thus results in the hybridization of the haptenylated probe and the desired target sequence such that a hybridized molecule having a double-stranded region is formed.

5 Simultaneously, or in the next step of the preferred method, this complex is "captured" using a hapten binding ligand molecule that has been bound to a solid support. Suitable hapten binding ligands include anti-hapten antibody (or antibody fragments), hapten receptor, etc. The choice of ligand will vary with the particular hapten employed. For example, when biotin is employed as the hapten, the hapten binding ligand is preferably avidin, streptavidin, or antibody or antibody fragments that bind biotin. Where the probe contains a homopolymeric region (e.g., poly-biotinylated dC), it is preferable to add a "counter-probe" of complementary sequence (e.g., where the probe has a poly-biotinylated dC homopolymeric region, the counter-probe may be a nucleic acid molecule having a poly-dG or poly-dC region). The addition of the counter-probe is optional, and serves to reduce the background extent to which undesired sequences are recovered. The use of such counter-probe is thus desirable when the level of undesirable species recovered by the probe is considered unacceptable.

15 Suitable solid supports include, but are not limited to, beads, tubes, or plates, which may be made of materials including, but not limited to, latex, glass, polystyrene, polypropylene or other plastic. Such supports can be 2-dimensional strips, beads, etc. A preferred support is a magnetic or paramagnetic bead (Seradyn, Indianapolis, IN). In a preferred embodiment, the capture of the hybridized haptenylated probe is initiated without the necessity for removing non-hybridized molecules.

20 Methods for effecting the attachment of the hapten binding ligand to the support are described by Hevey et al. (U.S. Patent No. 4,228,237) and by Kourilsky et al. (U.S. Patent No. 4,581,333). When a biotin hapten is employed, a paramagnetic-streptavidin conjugated bead, obtained from Life Technologies, Inc. (Gaithersburg, Maryland) or the Dynabead Streptavidin M-280 beads obtained from Dynal (Great Neck, NY) can be used as the ligand and support.

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The addition of the beads (or other support) to the reaction permits the haptenylated probe to bind to the hapten-binding ligand of the support. Such binding reactions are very strong. For example, the binding constant for the reaction between avidin and biotin is approximately 1,015 l/mole.

5 The very strong nature of this bond has been found to persist even when biotin is conjugated, by means of its carboxyl group, to another molecule, or when avidin is attached to another molecule.

As a consequence of such binding, any haptenylated probe that has hybridized to a desired target molecule will become bound to the support. In contrast, non-target molecules will remain unbound, and can be separated from the bound material by washing, filtration, centrifugation, sieving, or (in the case of paramagnetic or magnetic supports) by magnetic separation methods.

10 Most preferably, however, paramagnetic beads are used as the support, and a magnet is used to pull the paramagnetic beads out of solution, and the beads are washed with a suitable buffer (such as one containing Tris, EDTA, and NaCl). Such treatment removes the majority of non-target nucleic acid sequences that were originally present, and hence eliminates undesired non-selected single-stranded nucleic acid molecules from the reaction.

15 The specifically captured single-stranded target nucleic acid molecules (hybridized to the haptenylated probe) is then released from the probe by one or a combination of treatments, such as addition of an alkaline buffer, addition of one or more amino acid denaturants, heat, etc. Preferably, one or more amino acid denaturants or combinations thereof are used to release the nucleic acid molecules from the support bound probe. The releasing treatment is preferably selected such that the haptenylated probe remains attached to the support. The desired released target molecules are then isolated and may be subject to further selection. Such further selection may include additional probe hybridizations with one or more probes (the same or different than the probes used in the initial selection).

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Note that the hapten need not be covalently coupled to the probe-nucleic acid. The hapten may be linked, either covalently or non-covalently, to a molecule that non-covalently binds the probe molecule, e.g., a single-stranded DNA binding protein. The binding protein must  
5 bind tightly enough that significant quantities of it will not become disassociated from the probe molecules and bind to nucleic acid molecules of the sample.

This aspect of the present invention permits the recovery of a desired nucleic acid species from a mixture of nucleic acid molecules (i.e.,  
10 from a target mixture). The target mixture contemplated by the present invention will generally have more than 100 members, and typically more than 1,000, or even 10,000 members, 100,000 members or more. The methods of the present invention are thus capable of recovering a desired member of a target mixture even when such desired member is present at a  
15 concentration of less than 1%, 0.1%, 0.01%, 0.001%, 0.0001% or less (percentages are the ratio of the desired species per total number of different species present in the mixture).

#### **B. Enrichment/Selection of Larger or Full Length Desired Molecules**

In another preferred embodiment, larger or full-length desired  
20 nucleic acid molecules from a population of molecules may be obtained using the process of the invention. Thus, the invention provides a method to first select for desired target molecules (e.g., genes or gene fragments) and then allows for selection of larger or full-length target  
25 molecules (e.g., full-length genes). In this aspect of the invention, the subpopulation of desired nucleic acid molecules are separated according to size.

In accordance with the invention, size selection may be  
30 accomplished by standard gel electrophoresis techniques (agarose or acrylamide gel electrophoresis) and the larger or full-length molecules may be extracted from the gel. In a preferred aspect of the invention, prior to

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separation by size, the nucleic acid molecules are amplified by well known amplification techniques.

5 In one embodiment, the subpopulation of target nucleic acid molecules are contained in a vector which facilitates amplification of the nucleic acid molecules inserted into the vector. Such amplification may be accomplished by contacting the subpopulation of target nucleic acid molecules with a first probe which hybridizes to a portion of the vector and a second probe which hybridizes to a portion of the vector insert. Depending on the location of the probes used, amplification of either the 5' or the 3' portions of vector inserts in the population is accomplished. Upon separation by size, the invention thus provides enrichment for molecules having longer segments at the 5' or 3' terminus. Such longer segments may then be used to re-create or construct longer or full-length gene segments. For example, the 5' or the 3' larger segment may be sub-cloned to replace a shorter segment in a vector containing a desired nucleic acid molecule. Such replacement may be accomplished by well known restriction and ligation techniques.

15 Alternatively, amplification may be accomplished by using a first probe which is complementary to a portion of the vector at or near the 3' terminus of the vector insert and a second probe which is complementary to a portion of the vector at or near the 5' terminus of the vector insert. Amplification using such probes allows for complete amplification of the entire vector insert for each member of the population. Upon size selection, larger inserts or full-length segments of the desired nucleic acid molecule may be obtained for further processing. Typically, such amplification may require amplification of long templates. Amplification of long templates (5 to 12 Kb; Long PCR) may be accomplished by using a combination of a DNA polymerase lacking 3' exonuclease activity and a DNA polymerase having 3' exonuclease activity (see U.S. Patent 5,435,149). Such combination of polymerases are available commercially such as Elongase™ from Life Technologies, Inc. (Gaithersburg, Maryland).

25  
30 In another aspect of the invention, larger or full-length nucleic acid molecules may be selected by using a combination of amplification probes.

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In this embodiment, a first probe complementary to a portion of the vector sequence at or near the 3' terminus of the insert, a second probe complementary to a portion of the vector sequence at or near the 5' terminus of the insert, a third primer complementary to a first portion of the vector insert, and a fourth primer complementary to a second portion of the vector insert may be used (see Figure 3). Upon amplification using such primers, a first amplified region containing a 5' terminus of the vector insert and a second amplified region containing the 3' terminus of the insert is amplified. After amplification, both segments may be linked by an overlapped extension reaction (Horton *et al.*, *Gene* 77:61-68(1989); Jayaraman *et al.*, *Proc. Natl. Acad. Sci. (U.S.A.)* 88:4084-4088(1991)) in which the overlap of the two segments is used to join the two segments into a single segment. In this manner, the entire insert of the vector may be amplified without the need for long template amplification (above) or this amplification process may allow for amplification of extremely long inserts by combining long amplification with overlap extension reactions. After amplification, the larger or full-length inserts can be selected by size from the population.

This aspect of the invention is of particular interest for enriching for full-length genes obtained from a cDNA library. When preparing cDNA from the mRNA template, the first strand reaction typically provides a population of cDNA molecules (a portion of which are full-length) due to the failure of reverse transcriptase to completely synthesis cDNA from the mRNA template. The cDNA library comprises a population of cDNA molecules encoding significant numbers of genes (encoded by the tissue or cell from which the RNA was isolated) and as noted for each gene there is a subpopulation of cDNA molecules of varying sizes (some of which are full-length). The invention specifically provides a means to select a gene specific subpopulation (which then can be used for enrichment of full-length molecules) from the cDNA library. This aspect of the invention specifically comprises:

- (a) contacting a single-stranded cDNA library with one or more haptenylated nucleic acid probes comprising a nucleotide

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sequence complementary to a nucleotide sequence of one or more desired target molecules (e.g. gene specific probes);

(b) isolating said desired target molecules with one or more binding ligands conjugated to a support; and

5 (c) amplifying all or a portion of said desired target molecules and separating said amplified molecules according to size.

### C. Polymerase Enrichment/Selection of Desired Molecules

10 In another preferred embodiment, a polymerase enrichment/selection protocol can optionally be used to aid, or further aid, in effecting the isolation of a desired target molecules. In this embodiment, a nucleic acid primer molecule having a nucleotide sequence complementary to a region of the desired target nucleic acid molecule is introduced into the reaction. A polymerase and appropriate nucleotides are also added, and the reaction is incubated under conditions sufficient to permit the primer to

15 hybridize to the above-described single-stranded molecule (which is preferably a single stranded circular molecule), and to mediate the extension of the primer to form a double-stranded desired target nucleic acid molecule.

20 In one sub-embodiment, the primer molecule may have a nucleotide sequence that is complementary to the same region (or a subset or extension of the same region) as that which had been hybridized to the above-described probe. In such a case, the primer molecule maintains a selection for molecules of the initial sample that contains a single particular region (e.g., a promoter, enhancer, gene of interest, etc.).

25 Preferably, stringent hybridization conditions are used and the conversion of single stranded nucleic acid to double stranded nucleic acid is done at high temperature with a thermostable polymerase, e.g., Taq polymerase. In this case, because the hybridization and double-strand conversion are done under conditions favorable to correct hybrids, the conversion step further

30 enriches for or selects for the desired target molecules.

In an alternative sub-embodiment, the nucleotide sequence of the primer molecule is selected to be different from that of the probe, such that

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the primer molecule will hybridize to a region of the desired molecule other than the region that had been previously hybridized to the probe. This sub-embodiment permits one to select a subset of desired molecules that possess a further desired characteristic. For example, if the probe molecule hybridized to a particular enhancer element, the capture selection step described above would enrich for those members of the original mixture or library that contained the enhancer element. By employing a primer complementary to a particular receptor binding site, promoter element, gene sequence, terminator, etc., one would obtain double-stranded molecules that comprise that subset of the original mixture or library that contained both the enhancer element and the particular receptor binding site, promoter element, gene sequence, terminator, etc.

As indicated above, double-stranded nucleic acid molecules (e.g., DNA) transform more efficiently than single-stranded nucleic acid molecules, hence, by transforming bacteria or eukaryotic cells with the double-stranded molecules obtained from the first or second sub-embodiments, and then recovering nucleic acid molecules from the transformants, one is able to obtain a substantial enrichment for the desired target nucleic acid molecules.

In some cases, such as where the prevalence of desired target molecules is low, it may be desirable to eliminate undesired single-stranded non-target molecules that remain after the double-strand conversion of target molecules. This may be accomplished by conducting the template-dependent extension of the primer in the presence of at least one "nucleotide analog" (either in lieu of or in addition to the naturally occurring non-analog). A "nucleotide analog", as used herein, refers to a nucleotide which is not found in the target DNA or RNA that is the primer's template. For example, where the isolated target molecule is DNA, suitable nucleotide analogs include ribonucleotides, 5-methyldeoxycytosine, bromodeoxyuridine, 3-methyldeoxy-adenosine, 7-methyl-guanine, deoxyuridine, and 5,6-dihydro-5,6-dihydroxydeoxythymidine, etc. (see, Duncan, B.K., *The Enzymes* XIV:565-586 (1981)). Other nucleotide analogs will be evident to those of skill in the art. Where the template is

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RNA, deoxynucleotide triphosphates and their analogs are the preferred nucleotide analogs.

The presence of the nucleotide analog in the reaction will result in the production of a double-stranded molecule that contains incorporated analog bases. Such incorporation affects the ability of endonucleases and exonucleases to cleave or degrade the double-stranded molecule. Thus, if a primer is extended from a circular DNA template in the presence of a methylated nucleotide (for example, 5-methyl dCTP), the resulting double-stranded molecule, which contains incorporated 5-methyl C residues, is resistant to cleavage by many restriction endonucleases. *HhaI* is particularly preferred when used in conjunction with 5-methyl C residues, since it also degrades single-stranded DNA, the effect of incubation in the presence of such enzymes is to destroy most or all residual undesired non-target molecules present, and to thereby greatly enrich the concentration of the desired vector. Other nucleotide analogs that inhibit or block exonucleases or restriction endonucleases are 6-methyladenine, 5-methyl-guanine and 5-methylcytidine. Combinations of nucleotide analogs and suitable enzymes may be used in the invention and are known in the art (see, for example, Life Technologies<sup>TM</sup> 1993-1994 Catalogue and Reference Guide, Chapter 6, Life Technologies, Inc. (Gaithersburg, Maryland), herein incorporated by reference).

In a similar manner, where the source library was composed of single-stranded RNA vectors, the use of dNTPs (i.e. dATP, dTTP, dCTP, and dGTP) in the conversion step will render such molecules resistant to mung bean nuclease, or Bal-31 nuclease.

Although the foregoing discussion has emphasized the use of circular molecules, the methods of the present invention are fully amenable to the use of linear molecules. In such a case, the primer molecule (but not necessarily the probe molecule) is preferably selected such that it hybridizes to the 5' terminus of the target molecule. Such selection will permit the template-dependent extension of the molecule to produce a full length copy of the target molecule.

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Desirably, the recovered target molecules are then precipitated with organic solvents, and resuspended in buffer. The product may then be transformed or electroporated into recipient cells, for example by the method of Rubenstein *et al.* (*Nucl. Acids Res.* 18: 4833 (1990), herein incorporated by reference). Any recipient cell may be used, including prokaryotic or eukaryotic cells, although prokaryotic cells and bacteria, such as gram negative bacteria, are preferred. Particularly preferred gram negative bacteria include *E. coli*, *Salmonella*, *Klebsellia*, etc. Electrocompetent and chemically competent *E. coli* may be obtained from Life Technologies, Inc. (Gaithersburg, Maryland).

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

#### EXAMPLE 1

#### Method for Isolating Desired Target Molecules

##### Preparation of single-stranded DNA

A preferred method for isolating a desired target molecule employs a library (preferably a cDNA library) in a single-stranded phagemid, such as M13 or preferably, vectors such as pSPORT 1, pCMV•SPORT, pZL1 ( $\lambda$ Ziplox®), and BlueScript SK (+). In a typical reaction, 5  $\mu$ g of double-stranded phagemid and 2  $\mu$ l of 10X Gene II buffer (200 mM Tris (pH 8.0), 800 mM NaCl, 25 mM MgCl<sub>2</sub>, 20 mM  $\beta$ -mercaptoethanol, 50% glycerol, 50mg/ml BSA) was incubated with 1  $\mu$ l of Gene II (10 units/ $\mu$ l), in a reaction volume of 20  $\mu$ l. The reaction mixture was vortexed and then centrifuged at room temperature for 2 seconds at 14,000  $\times$  g prior to being incubated at 30°C for 30 minutes. The reaction was terminated by heating the mixture at 65°C for 5 minutes and then immediately thereafter chilling the mixture on ice for 1 minute. After the reaction was terminated, 1  $\mu$ l of the mixture was transferred to a new microcentrifuge tube containing 9  $\mu$ l TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and 2  $\mu$ l of 6X gel loading

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buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll (type 400 in water)) and retained at 4°C for later agarose gel analysis.

To the remaining 19  $\mu$ l of reaction mixture, 2  $\mu$ l of Exonuclease III (65 units/ $\mu$ l) was added. Before incubation, the reaction mixture was vortexed and centrifuged at room temperature for 2 seconds at 14,000 x g. The reaction mixture was then incubated at 37°C for 1 hour and then stored on ice. 1  $\mu$ l of the reaction mixture was transferred to new microcentrifuge tube containing 9  $\mu$ l TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) and 2  $\mu$ l of gel loading dye and retained at 4°C for later agarose gel analysis.

The samples retained for agarose gel analysis were loaded on a 0.8% agarose gel in 1X TAE buffer (40 mM Tris-acetate (pH 8.3), 1 mM EDTA) to determine whether the double stranded DNA was converted to single stranded DNA by the Gene II/Exonuclease III digestion. Typically, more than 50% of the supercoiled DNA should be nicked by the Gene II protein and migrate as relaxed circular DNA and the nicked form of the double-stranded DNA generated by Gene II treatment should be completely converted to single-stranded DNA after Exonuclease III digestion. If the double stranded DNA (ds-DNA) is converted to single stranded (ss-DNA), then hybridization with the probe is performed (see below).

#### Preparation of Biotinylated Oligonucleotides

The oligonucleotide probes were biotin-labeled using biotin-14-dCTP and terminal deoxynucleotidyl transferase (TdT) as described by Flickinger, J.L. *et al.* (*Nucleic Acids Res.* 20: 2382 (1992)) with some modifications. In a typical reaction,  $\approx$ 3  $\mu$ g of oligonucleotides (16-25-mer), 5  $\mu$ l of 5X TdT buffer, 5  $\mu$ l of biotin-14-dCTP (5mM) and 2  $\mu$ l of TdT in a reaction volume of 25  $\mu$ l was incubated at 30°C for 1 hour. The reaction was terminated by precipitating the probes with 1  $\mu$ l of glycogen (20  $\mu$ g/ $\mu$ l), 26  $\mu$ l 1M Tris-HCl (pH 7.5) and 120  $\mu$ l of ethanol and storing on dry ice for 10 minutes. After centrifugation at 4°C for 30 minutes at 14,000 x g, the probes were rinsed with 200  $\mu$ l of 70% ethanol (-20°C) and centrifuged for 2 minutes at 14,000 x g at room temperature. The probes were air-dried and dissolved in 20  $\mu$ l of TE. To determine the labeling efficiency and the concentration of the labeled probe, 4  $\mu$ l of labeled products were resuspended in an equal

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5 volume of 90% formamide, 50 mM Tris-base, 45 mM boric acid, 0.5 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol. The probes were electrophoresed along with a known amount of the starting material on 16% denaturing PAGE. The gel was stained in an ethidium bromide solution (0.5  $\mu\text{g}/\text{ml}$ ) for 15 minutes, and photographed.

### Hybrid Selection

10 The hybridization was performed by the following procedure: to the remaining 20  $\mu\text{l}$  of Gene II/Exonuclease III treated DNA was added and mixed 7.0  $\mu\text{l}$  of 4X Hybridization Buffer (100 mM HEPES (pH 7.5), 2 mM EDTA, 0.2% SDS). The mixture was mixed by repeat pipeting. The DNA was denatured at 90°C for 1 minute and immediately chilled in ice water for 1 minute. 1  $\mu\text{l}$  (20 ng) of biotin-probe was added to the DNA mixture and the mixture was incubated at 37°C for 1 hour.

15 Before binding the hybrids to the streptavidin beads, 45  $\mu\text{l}$  of the streptavidin coated paramagnetic beads (Life Technologies, Inc., Gaithersburg, Maryland) were washed once with 100  $\mu\text{l}$  TE. The paramagnetic beads were resuspended in 30  $\mu\text{l}$  of TE.

20 After incubating the reaction mixture for 1 hour, the reaction mixture was centrifuged for 2 seconds at 14,000  $\times$  g. 30  $\mu\text{l}$  of resuspended beads was added to the hybridization mixture (27 ml) and mixed well by gentle pipeting. The mixture was incubated at room temperature for 30 minutes with occasional mixing by gently tapping the tube. The paramagnetic beads were separated from the DNA by inserting the tube into the magnet and washed 4 times with 100  $\mu\text{l}$  of wash buffer (10 mM Tris (pH 7.5), 1 mM EDTA).

25 Finally, the paramagnetic beads were resuspended in 10  $\mu\text{l}$  of 1X elution buffer (10 mM glycine) and incubated at room temperature for five minutes while being gently agitated. The supernatant was then removed and retained in a new tube while the beads were resuspended in 7  $\mu\text{l}$  of elution buffer. The tube containing the resuspended beads was inserted into the magnet for five minutes and the aqueous phases were pooled (26  $\mu\text{l}$  total). The tube containing the pooled supernatants was inserted into the magnet for 10 minutes to eliminate any remaining beads.

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### Repair of Single-Stranded DNA

5 A DNA repair mix containing 1  $\mu$ l (50 ng) of unlabeled primer, 17  $\mu$ l of the eluted single-stranded DNA, 0.5  $\mu$ l dNTP mix (10 mM), 0.5  $\mu$ l repair enzyme (DynaZyme, 2 units/ $\mu$ l), 2.0  $\mu$ l of 10X repair buffer (100 mM Tris (pH 8.8 at 25°C), 15 mM MgCl<sub>2</sub>, 500 mM KCl, 1% Triton X-100) was incubated at 90°C for 1 minute, 55°C for 30 seconds and then 70°C for an additional 15 minutes. Following these incubations, the reaction mixture was centrifuged for 2 seconds at 14,000 x g. After repair, the double-stranded DNA was stored at -20°C.

### 10 Detection of the Target Gene

15 The repaired DNA is used to transform *E. coli* bacteria by chemical transformation or electroporation using techniques well known to those of ordinary skill in the art. For transformation, cells obtained from Life Technologies, Inc. are used according to the following procedure: UltraMax competent cells are removed from -70°C and thawed on wet ice. Immediately after thawing, the cells are gently mixed and 100  $\mu$ l of competent cells are aliquoted into chilled polypropylene tubes. To determine transformation efficiency, 5  $\mu$ l (0.05 ng) control DNA to one tube containing 100  $\mu$ l competent cells. For each captured or repaired DNA reaction, mix 3  $\mu$ l of the repaired DNA into an individual tube of cells (store the remainder of the DNA reaction at -20°C) and incubate on ice for 20 30 minutes. The cells are then heat shocked for 45 seconds in a 42°C water bath without shaking and then stored on ice for 2 minutes. Following these incubations, 0.9 ml of S.O.C. medium is added and the cells are 25 shaken at 225 rpm for 1 hour at 37°C. For the control plasmid, the cells are diluted (1:400) and 100  $\mu$ l of the diluted cells are then spread on LB or YT plates containing 100  $\mu$ g/ml ampicillin. For the captured or repaired cDNA samples, plate 100  $\mu$ l and 200  $\mu$ l aliquots onto LB plates containing 100  $\mu$ g/ml ampicillin (e.g. pSPORT vector). The remainder of the cells are 30 centrifuged for 15 seconds in an autoclaved 1.5 ml microfuge tube, the supernatant is discarded, the cells are resuspended in 200  $\mu$ l of S.O.C. medium and plated onto an ampicillin plate. The plates are incubated

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overnight in a 37°C incubator. For electroporation, electrocompetent cells (e.g. DH10B) were obtained from Life Technologies, Inc. and transformed according the procedure provided by the manufacturer (see GeneTrapper Manual). After transformation or electroporation, the target colony can be detected by the PCR, colony hybridization or cycle sequencing approach.

5 Preferably the target gene is identified using PCR essentially as follows. The repaired DNA is used to transform *E. coli* bacteria. The resulting library is referred to as an enriched library. Each individual colony is added to an eppendorf tube containing 20 µl of 1X PCR buffer (50 mM KCl, 20 mM Tris-HCl (pH 8.4)), 0.2 mM dNTP mix, 0.2 µM primers, 1.5 mM MgCl<sub>2</sub> and 0.5 units Taq DNA polymerase. The tubes are placed in a thermal cycler prewarmed to 94°C. PCR is performed using the following program: 1 cycle: 94°C/2 minutes; 30 cycles of 94°C/30 seconds, 55°C/30 seconds, 72°C/2 minutes. After PCR, the presence of specific amplified products is evaluated by gel electrophoresis of an aliquot of the reaction mixture. The presence of a PCR product of the correct size confirms the presence of a desired clone.

## EXAMPLE 2

### Alternative Method for Isolating Desired Target Molecules

20 An alternative method for isolating a desired target molecule employs a library or mixture of a single-stranded phagemid, such as M13. In such a method, the single-stranded phagemid is introduced into an *ung dut* mutant of *E. coli* (Kunkel, T.A., U.S. Patent No. 4,873,192; Longo, M.C. *et al.*, *Gene* 93:125-128 (1990); Hartley, U.S. Patent No. 5,035,966; all herein incorporated by reference). The "+" strand of phagemids grown in such mutants contains deoxyuridine (dUTP), and can be recovered from the packaged virion. Thus, the use of such mutants permits the isolation of a library or mixture that comprises single-stranded DNA molecules which contain dU residues (Kunkel, T.A., U.S. Patent No. 4,873,192).

30 The recovered DNA can then be optionally isolated via a capture step, or directly processed using a nuclease enrichment step.

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5 If a capture step is to be conducted, the dU-containing strands are incubated in the presence of a complementary biotinylated probe. The probe, and any hybridized DNA is then recovered by permitting the biotin to bind to avidin or strepavidin coated paramagnetic beads, and then recovering the beads from solution using a magnet. The library or mixture is recovered from the beads by denaturation of the hybridized molecules.

10 The recovered single-stranded DNA is then incubated in the presence of a complementary primer, dATP, dTTP, dCTP, and dGTP and under conditions sufficient to permit the extension of the primer. Such extension thus creates a sample that contains single-stranded dU-containing molecules and double-stranded dU/dT hybrid (desired target) molecules.

15 Although the triphosphate form of deoxyuridine, dUTP, is present in living organisms as a metabolic intermediate, it is rarely incorporated into DNA. When dUTP is incorporated into DNA, the resulting deoxyuridine can be promptly removed in vivo by the enzyme uracil DNA glycosylase (UDG) (Kunkel, U.S. Patent No. 4,873,192; and Duncan, B.K., The Enzymes XIV:565-586 (1981), both references herein incorporated by reference in their entirety).

20 In this embodiment of the present invention, the mixture of molecules is then treated, either in vivo or in vitro with UDG. Such treatment destroys all of the single-stranded, non-desired, non-target molecules in the sample. It further destroys the "+" strand of all of the double-stranded desired target molecules.

25 The sample is therefore then either directly transformed into E. coli to permit the isolation of the target molecule or incubated in the presence of a primer molecule that is capable of hybridizing to the "-" strand of the phagemid. Such incubation is under conditions suitable for mediating the template-dependent extension of the primer. Hence, such incubation produces double-stranded molecules that have the sequence of the desired target molecules, and thereby permit the isolation of the target molecule.

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**EXAMPLE 3****Alternative Method for Preparation of Single-Stranded DNA**

The large scale preparation of single-stranded phagemid cDNA library may be made as described previously (Gruber, C.E. *et al.*, *Focus* 15: 59-65 (1993), herein incorporated by reference).

**EXAMPLE 4****Alternative Method for Preparation of Biotinylated Oligonucleotides**

The oligonucleotide probes were biotin-labeled using biotin-14-dCTP and terminal deoxynucleotidyl transferase (TdT) as described by Flickinger, J.L. *et al.* (*Nucleic Acids Res.* 20: 2382 (1992)) with the following minor modifications. In a typical reaction, 0.3-0.5 nmol ( $\approx 5 \mu\text{g}$ ) of oligonucleotides (21-25-mer), 500  $\mu\text{M}$  of biotin-14-dCTP and 60 units of TdT in 50  $\mu\text{l}$  of 1X tailing buffer (100 mM potassium cacodylate (pH 7.2), 2 mM  $\text{CoCl}_2$  and 200  $\mu\text{M}$  DTT) are incubated at 37°C for 15 minutes. The reaction is terminated by adding 2  $\mu\text{l}$  of 0.25 M EDTA. The labeled probes are precipitated by adding an equal volume (52  $\mu\text{l}$ ) of 1 M Tris buffer (pH 7.5), 10  $\mu\text{g}$  glycogen as carrier, and 2.5 volumes (260  $\mu\text{l}$ ) of ethanol, and stored on dry ice for 10 minutes. After centrifugation at 4°C for 10 minutes, the probes are rinsed with 100  $\mu\text{l}$  of 75% ethanol and centrifuged for 2 minutes. The probes are air-dried and dissolved in 10  $\mu\text{l}$  of TE. To determine the labeling efficiency and the concentration of the labeled probe, 2  $\mu\text{l}$  of labeled products are resuspended in an equal volume of sequencing reaction stop buffer (95% (v/v) formamide, 10 mM EDTA (pH 8.0), 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol), heated at 95°C for 1 minute and chilled on ice. The probes are electrophoresed along with a known amount of the starting material on 16% denaturing PAGE. The gel is stained in an ethidium bromide solution (0.5  $\mu\text{g}/\text{ml}$ ) for 15 minutes, and photographed. Typically, more than 95% of the oligonucleotide will be labeled. The concentration of the labeled probes is determined by the comparison to the known starting material.

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**EXAMPLE 5**  
**Alternative Method for Hybrid Selection**

The hybridization is performed by the following procedure: 1-10  $\mu\text{g}$  of single-stranded target library DNA is diluted with 10  $\mu\text{l}$  of dilution buffer (100 mM HEPES (pH 7.5), 2 mM EDTA and 0.2% SDS) to a final volume of 19  $\mu\text{l}$  in a 5 ml Falcon tube. The DNA is denatured at 95°C for 1 minute and immediately chilled in ice water for 5 minutes. 1  $\mu\text{l}$  (20 ng) of biotin-probe is added to the DNA mixture, followed by the addition of 5  $\mu\text{l}$  of 5 M NaCl. The hybridization mixture is incubated at 42°C with continuous shaking (200 rpm) in a culture incubator for 24 hours. Before binding the hybrids to the streptavidin, 50  $\mu\text{l}$  of the streptavidin coated paramagnetic beads (DYNAL) are washed once with 1X binding buffer (10 mM TRIS (pH 7.5), 1 mM EDTA and 1 M NaCl) by following the manufacturer's instructions. The paramagnetic beads are resuspended in 20  $\mu\text{l}$  of 1X binding buffer. The hybridization mixture is added to the resuspended beads and mixed well. The mixture is incubated at room temperature for 1 hour with occasional mixing by gently tipping the tube. The paramagnetic beads are separated from the DNA bulk by inserting the tube into the magnet, and washed 6 times with the washing buffer (10 mM Tris (pH 7.5), 1 mM EDTA and 500 mM NaCl). Finally, the paramagnetic beads are resuspended in 20  $\mu\text{l}$  of 30% formamide in TE buffer. The selected DNA is released by heating the beads at 65°C for 5 minutes. The tube is inserted into the magnet, and the aqueous phase is transferred to a new tube. The beads are washed once with 15  $\mu\text{l}$  of TE buffer, and the aqueous phases are pooled. The selected DNA is precipitated with 0.5 volumes of 7.5 M ammonium acetate, 10  $\mu\text{g}$  of glycogen, and 2.5 volumes of ethanol. The DNA pellet is dissolved in 5-10  $\mu\text{l}$  of TE buffer. An aliquot (1  $\mu\text{l}$ ) is used for electroporation to determine the hybrid selection efficiency.

**EXAMPLE 6**  
**Alternative Method for Repair of Single-Stranded DNA**

The remainder of the selected single-stranded DNA is converted to double-stranded DNA before electroporation as described by Rubenstein *et*

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al. (*Nucl. Acids Res.* 18: 4833 (1990)) with some modifications. The reaction is carried out in 30  $\mu$ l containing the selected single-stranded DNA, 250 ng of unlabeled primer, 300  $\mu$ M each dTTP, dGTP, dATP and 5-methyl dCTP, Taq DNA polymerase buffer and 2 units of Taq DNA polymerase. After repair, the mixture is extracted once with phenol:chloroform. The organic phase is back-extracted with 15  $\mu$ l of TE, the aqueous phases are pooled and ethanol precipitated. The pellet is rinsed with 100  $\mu$ l of 75% ethanol and dried. The repaired DNA is dissolved in 5-10  $\mu$ l of TE and digested with *Hha*I for 2 hours at 37°C. After digestion, the mixture is extracted once with phenol:chloroform, ethanol precipitated and dissolved in 5-10  $\mu$ l of TE.

#### EXAMPLE 7

##### Methods for Enrichment of Full Length cDNA Molecules using PCR Amplification

In another embodiment of the present invention, the present invention may be used to preferentially isolate cDNA molecules containing larger DNA inserts. A cDNA library is generated according to the procedure set forth in Example 3. To select for target molecules from the cDNA library, the selection method of Example 1 is used. The isolated target molecules are then subjected to size enrichment.

For this purpose, two PCR reactions are set up and carried out essentially as set forth in Example 1. Each PCR reaction uses a pair of PCR primers, one complementary to the target sequence and one complementary to the vector sequence (see Figure 3, steps 1 and 2). The PCR products are then used in an overlap extension reaction (Horton *et al.*, *Gene* 77: 61-68 (1989); Jayaraman *et al.*, *Proc. Natl. Acad. Sci. (USA)* 88:4084-4088 (1991)) (see Figure 3, step 3). The products of primer extension reaction are then separated by gel electrophoresis and may be then cloned into an appropriate vector, e.g. a TA vector, prior to transformation of an appropriate host cell. Colonies after transformation are tested for the presence of the target sequence by colony PCR and selected colonies may be tested by DNA sequencing.

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**EXAMPLE 8**  
**Comparison of Elution Buffers**

A novel elution buffer was developed to remove the biotinylated capture probe hybridized to the target nucleic acid molecule (e.g., cDNA molecule). Originally a 30% formamide/TE (pH 8.0) buffer was used which required an ethanol precipitation following its use. A novel elution buffer containing 10 mM glycine was shown to be effective and when compared with the formamide/TE buffer produced more colonies and a higher percentage of these were positive for the CAT plasmid target that was mixed in the cDNA library at a ratio of 1:50,000.

Elution Buffer	# of ampicillin resistant colonies	# of chloroamphenicol resistant colonies	% CAT
30% formamide/TE pH 8.0	75	41	55
10 mM glycine	250	162	65

In addition to 10 mM glycine, 15 other amino acids/amino acid analogs were tested and shown to be effective as an elution buffer, including the following amino acids: alanine, arginine, asparagine, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and the nitrogenous base, imidazole (see Example 11).

**EXAMPLE 9**  
**Use of dP and dK Containing Degenerate Oligonucleotides**

A comparison was made using the procedure of Example 1 using degenerate biotinylated probes containing dP and dK. These probes had a degeneracy of 1,024 with the same oligonucleotide in which dK had been substituted for the A/G degenerate position, dP for the C/T degenerate position and dP/dK for all four nucleotides. In effect, each substitution

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with dP or dK reduces the complexity of the oligonucleotide population by a factor of 2.

5 When a pSPORT1 plasmid containing the chloramphenicol (CAT) gene is mixed at 1:50,000 with a cDNA library, the percent positive (ie., CAT clones) increases 4-fold favoring the dP-dK substituted oligonucleotide as depicted in Table 2.

	# of ampicillin resistant colonies	# of chloramphenicol resistant colonies	% CAT
D1024-expt1	308	31	10
D1024-expt2	346	34	9.8
D1024-PK-expt1	126	50	39.7
D1024-PK-expt2	151	65	43

10 Oligonucleotide D1024 (GTN TG(T/C) GA(T/C) GGN TT(T/C) CA(T/C) GTN GG) (Seq ID NO 1) has a degeneracy of 1024. The sequences represented by oligonucleotide D1024-PK, which has a degeneracy of 8, are depicted in Table 3.

SEQ ID NO	Sequence
2	GTK TGP GAP GGK TTP CAP GTK GG
3	GTK TGP GAP GGK TTP CAP GTP GG
4	GTK TGP GAP GGP TTP CAP GTK GG
5	GTP TGP GAP GGK TTP CAP GTK GG
6	GTP TGP GAP GGK TTP CAP GTP GG
7	GTK TGP GAP GGP TTP CAP GTP GG
8	GTP TGP GAP GGP TTP CAP GTK GG
9	GTP TGP GAP GGP TTP CAP GTP GG

**EXAMPLE 10**  
**Use of 5-methyl Deoxycytosine (5mC)/*HhaI* in the Repair Reaction**

Experiments have reproducibly shown that the inclusion of the methylated nucleotide 5mC in combination with the enzyme *HhaI* can reduce the number of background colonies. Using a mixture of the CAT plasmid described in Example 9 above with a cDNA library at 1:50,000, experiments as essentially described in Example 1 (with or without 5 mC) were used to compare the effect of the repair reaction using nuclease resistant analogs. The data presented in Table 3 demonstrates that the background can be reduced when the 5 mC protocol, described below, is used.

The DNA primer/repair mixture for each capture reaction was prepared, on ice, by adding to the captured DNA (26  $\mu$ l) tube 1  $\mu$ l of unbiotinylated oligonucleotide (50 ng), 0.5  $\mu$ l of dATP, dGTP, dTTP and 5-methyl d-CTP mix (10 mM each), 3  $\mu$ l of 10X Repair Buffer, 0.5  $\mu$ l (1 unit) Repair Enzyme. The DNA primer/repair mixture was mixed by repeat pipetting and centrifuged at room temperature for 2 seconds at 14,000 x g. After centrifugation, the DNA primer/repair mixture was incubated at 85°C for 1 minute, incubated at 55°C for 30 seconds, and incubated at 70°C for 15 minutes to allow for primer extension. After incubating for 15 minutes at 70°C, the tubes were centrifuged for 2 seconds and cooled to room temperature. After the tubes had cooled to room temperature, 1  $\mu$ l of *HhaI* (0.25-0.5 units) was added to the reaction mixture, mixed, centrifuged for 2 seconds at 14,000 x g, and incubated at 37°C for 30 minutes. After the 30 minute incubation, the DNA was transferred to a fresh tube and precipitated by adding 1  $\mu$ l of glycogen (20  $\mu$ g), 4  $\mu$ l of 3M sodium acetate, and 90  $\mu$ l of ethanol. The tubes were incubated on ice for at least 10 minutes and then centrifuged for 30 minutes at 4°C. The supernatant was decanted and the DNA pellet was washed with 100  $\mu$ l of 70% ethanol (-20°C) and centrifuged at room temperature for 2 minutes. The ethanol was decanted, the DNA pellet was dried at room temperature for 5-10 minutes, and the DNA pellet was resuspended in 10  $\mu$ l of TE. DH10B competent cells were electroporated with 2  $\mu$ l of each sample.

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Table 3			
	# of ampicillin resistant colonies	# of chloramphenicol resistant colonies	% CAT
no 5mC-expt1	210	138	65.2
5mC-expt2	124	76	61.5
no 5mC-expt1	63	58	92
5mC-expt2	71	67	95

### EXAMPLE 11

#### Assay For Determining Denaturation Of Double-Stranded Nucleic Acid Molecules With Amino Acid Denaturants

5

A protocol was developed to determine the ability of amino acid denaturants to denature or separate double-stranded nucleic acid molecules to form single-stranded nucleic acid molecules (e.g., double-stranded DNA to form single-stranded DNA molecules). In this method, pSPORT I-CAT DNA is used as a template for partial repair with DNA polymerase and radio-labeled nucleotides. Specifically, 0.7  $\mu$ g of single-stranded pSPORT I-CAT DNA was partially repaired with primer and P32-dCTP/dNTPs as described in Example 1 (repair of single-stranded DNA), except that the denaturation at 90°C and the incubation at 70°C was performed for 4 minutes rather than 15 minutes. After the partial repair reaction, 25 ng of P32-labeled pSPORT ICAT DNA in 17 $\mu$ l of 1X GENE II buffer was hybridized to a biotinylated probe (SEQ ID NO 10) (GAC CGT TCA GCT GGA TAT TAC GGC C) and the hybridized molecules were captured on strepavidan magnetic beads as described in Example 1 (hybrid selection). The beads were washed 4 times with wash buffer (10 mM Tris (pH 7.5), 1 mM EDTA) and the hybridized molecules were then tested with amino acid solutions to determine the effect of the amino acid solutions as denaturants.

In this assay, the ability of the amino acid denaturants to remove radioactivity from the solid support (e.g. the beads) indicated that the

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amino acid denaturants has the ability to denature or separate the double-stranded nucleic acid molecules. Tests were performed using 10 mM concentrations of amino acid in solution. A number of amino acid solutions (10 mM) acted as denaturants in this assay. These amino acid denaturants include glycine, alanine, asparagine, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine and imidazole. As will be appreciated, other amino acids, their derivatives or analogs as well as polyamino acids (their derivatives or analogs) may be used as denaturants in accordance with the invention. The concentrations of such amino acid denaturants which are optimal for denaturation may be determined using the above assay by one of ordinary skill in the art.

#### EXAMPLE 12

##### Use of Degenerate Kozak Consensus Sequence Oligonucleotides to Isolate cDNA Clones Containing the Translation Initiation Codon

In another embodiment of the present invention, the present invention may be used to preferentially isolate cDNA molecules that contain the 5' terminus including the translation initiation codon. This is accomplished by developing degenerate oligonucleotide to the Kozak sequence which includes the translation initiation codon and extends 5' approximately 13 nucleotides (Kozak, M, *Nucleic Acids Res.* 8:125-32 (1987); Kozak, M, *J. Biol. Chem* 266:19867-70 (1991)). The consensus sequence for initiation of translation by eukaryotic ribosomes is GCC GCC A<sup>-3</sup> /GCC A<sup>1</sup>UGG<sup>4</sup> (SEQ ID NO 11), Kozak, M, *Nucleic Acids Res.* 8:125-32 (1987); Kozak, M, *J. Biol. Chem* 266:19867-70 (1991), herein incorporated by reference; Sambrook *et al.*, 16.16, In *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Press (1989), herein incorporated by reference. Two approaches can be attempted to enrich for the presence of the 5' terminus including the translation start codon. In the first, the degerenate Kozak oligonucleotide prbe can be used to enrich by GeneTrapper for 5' sequences followed by the use of a gene-specific GeneTrapper probe. Alternatively, a gene-specific GeneTrapper probe can be applied to a phagemid cDNA

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library using GeneTrapper followed by the use of a degenerate Kozak oligonucleotide probe. In both cases, the percentage of clones that contain the 5' terminus including the translation initiation codon should be enriched. This method will be especially useful for clones derived from  
5 longer mRNAs (i.e., greater than 5 Kb).

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles  
10 of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

15 All patents, patent applications and publications referenced herein, are incorporated by reference in their entirety.

- 4 3 -

## SEQUENCE LISTING

## 5 (1) GENERAL INFORMATION

(i) APPLICANT: Nisson, Paul  
Jessee, Joel  
Li, Wu-bo

10

(ii) TITLE OF THE INVENTION: Method of Nucleic Acid Sequence  
Selection

(iii) NUMBER OF SEQUENCES: 11

15

(iv) CORRESPONDENCE ADDRESS:

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20

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(E) COUNTRY: USA

(F) ZIP: 20004-2402

(v) COMPUTER READABLE FORM:

25

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: DOS

(D) SOFTWARE: FastSEQ for Windows Version 2.0

30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

35

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

40

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(C) REFERENCE/DOCKET NUMBER: 04227.0026

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(A) TELEPHONE: 202 383-7451

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(2) INFORMATION FOR SEQ ID NO:1:

10

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

15 (D) TOPOLOGY: linear

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

20 GTNTGYGAYG GNTTYCAYGT NGG

23

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 19 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

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

GTKTGGAGGK TTCAGTKGG

19

35 (2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

40 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- 4 5 -

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

GTKTGGAGGK TTCAGTGG 18

5 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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

15 GTKTGGAGGT TCAGTKGG 18

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

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

25  
30 GTTGGAGGKT TCAGTKGG 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35  
40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTTGGAGGKT TCAGTGG 17

-46-

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 17 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

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

GTKTGGAGGT TCAGTGG 17

## (2) INFORMATION FOR SEQ ID NO:8:

15

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
20 (D) TOPOLOGY: linear

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

25 GTTGGAGGTT CAGTKGG 17

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 16 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

35

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

GTTGGAGGTT CAGTGG 16

40

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs

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

5

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

GACCGTTCAG CTGGATATTA CGGCC

25

10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

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

GCCGCCAGCC AUGG

14

**WHAT IS CLAIMED IS:**

1. A method for denaturing or separating double-stranded nucleic acid molecules, said method comprising contacting one or more double-stranded nucleic acid molecules with one or more amino acid denaturants under conditions sufficient to form single-stranded nucleic acid molecules.  
5
2. The method of claim 1, wherein said amino acid denaturants are selected from the group consisting of one or more amino acids, derivatives, analogs thereof or combinations thereof, and one or more polyamino acids, derivatives, analogs thereof or combinations thereof.  
10
3. The method of claim 2, wherein said polyamino acids comprise two or more amino acids or derivatives or analogs thereof.
4. The method of claim 2, wherein said amino acids are selected from the group consisting of glycine, alanine, arginine, asparagine, glutamine, isoleucine, leucine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and imidazole.  
15
5. The method of claim 4, wherein said amino acid is glycine.
6. The method of claim 1, wherein the concentration of said amino acid denaturants ranges from about 1 mM to about 500 mM.  
20
7. The method of claim 6, wherein said concentration ranges from about 5 mM to about 50 mM.
8. The method of claim 7, wherein said concentration is about 10 mM.
9. A method of recovering one or more desired target nucleic acid molecules from a population of nucleic acid molecules comprising:  
25
  - a) contacting said population with one or more hypentylated probes, under conditions sufficient to permit said probe to

hybridize to said desired target molecules thereby forming one or more hybridized molecules; and

- b) isolating said desired target nucleic acid molecules from said probes by contacting said hybridized molecules with one or more amino acid denaturants.

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10. The method of claim 9, wherein said population of nucleic acid molecules is single-stranded DNA.

11. The method of claim 10, wherein said single-stranded DNA is circular.

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12. The method of claim 10, wherein said single-stranded DNA is linear.

13. The method of claim 10, wherein said single-stranded DNA is selected from the group consisting of single-stranded plasmids, single-stranded cosmids, and single-stranded phagemids.

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14. The method of claim 9, wherein said population of nucleic acid molecules is a cDNA library.

15. The method of claim 9, wherein said haptenylated probes are bound to a support.

16. The method of claim 15, wherein said support comprises one or more binding ligands.

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17. The method of claim 16, wherein said probes are bound to said support by a ligand-hapten interaction.

18. The method of claim 9, wherein said population of nucleic acid molecules is double-stranded DNA.

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19. The method of claim 18, further comprising treating said double-stranded nucleic acid molecules under conditions sufficient to render such molecules single-stranded.

20. The method of claim 19, wherein said treatment comprises contacting said double-stranded nucleic acid molecule with one or more amino acid denaturants.
- 5 21. The method of claim 19, wherein said treatment comprises degradation of one strand of said double-stranded nucleic acid molecules.
22. The method of claim 21, wherein said degradation comprises the use of Gene II protein and Exonuclease III.
- 10 23. The method of claim 9, further comprising (c) incubating said isolated desired target nucleic acid molecules under conditions sufficient to synthesize a nucleic acid molecule complementary to said desired target molecules, thereby forming double-stranded nucleic acid molecules.
- 15 24. The method of claim 23, wherein said conditions comprise the use of one or more primer nucleic acid molecules and one or more nucleotides.
25. The method of claim 24, wherein said nucleotides confer nuclease resistance to said synthesized nucleic acid molecule.
- 20 26. The method of claim 25, wherein said nucleotides are nucleotide analogs.
27. The method of claim 26, wherein said nucleotide analogs are a methylated nucleotides.
28. The method of claim 27, wherein said methylated nucleotides are 5-methyldeoxycytosine.
- 25 29. The method of claim 25, further comprising digesting said double-stranded nucleic acid molecules with one or more nuclease.

30. The method of claim 29, further comprising transforming said digested molecules into one or more host cells.
31. The method of claim 23, further comprising transforming said double-stranded molecules into one or more host cells.
- 5 32. The method of claim 9, wherein said probes are degenerate probes.
33. The method of claim 32, wherein said degenerate probes comprise one or more universal nucleotides.
34. The method of claim 33, wherein said degenerate probes comprise one or more nucleotides selected from the group consisting of dP and dK.
- 10
35. The method of claim 9, further comprising enriching for larger or full-length desired nucleic acid molecules.
36. The method of claim 35, wherein said enrichment comprises separating the desired nucleic acid molecules according to size.
- 15 37. The method of claim 36, wherein said method comprises amplifying the desired nucleic acid molecules prior to size separation.
38. The method of claim 9, wherein said probes comprise a Kozac sequence.
39. The method of claim 38, wherein said probes are degenerate probes.
- 20 40. The method of claim 32, wherein said degenerate probes are to a Kozac sequence.

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OVERVIEW OF THE cDNA POSITIVE SELECTION SYSTEM

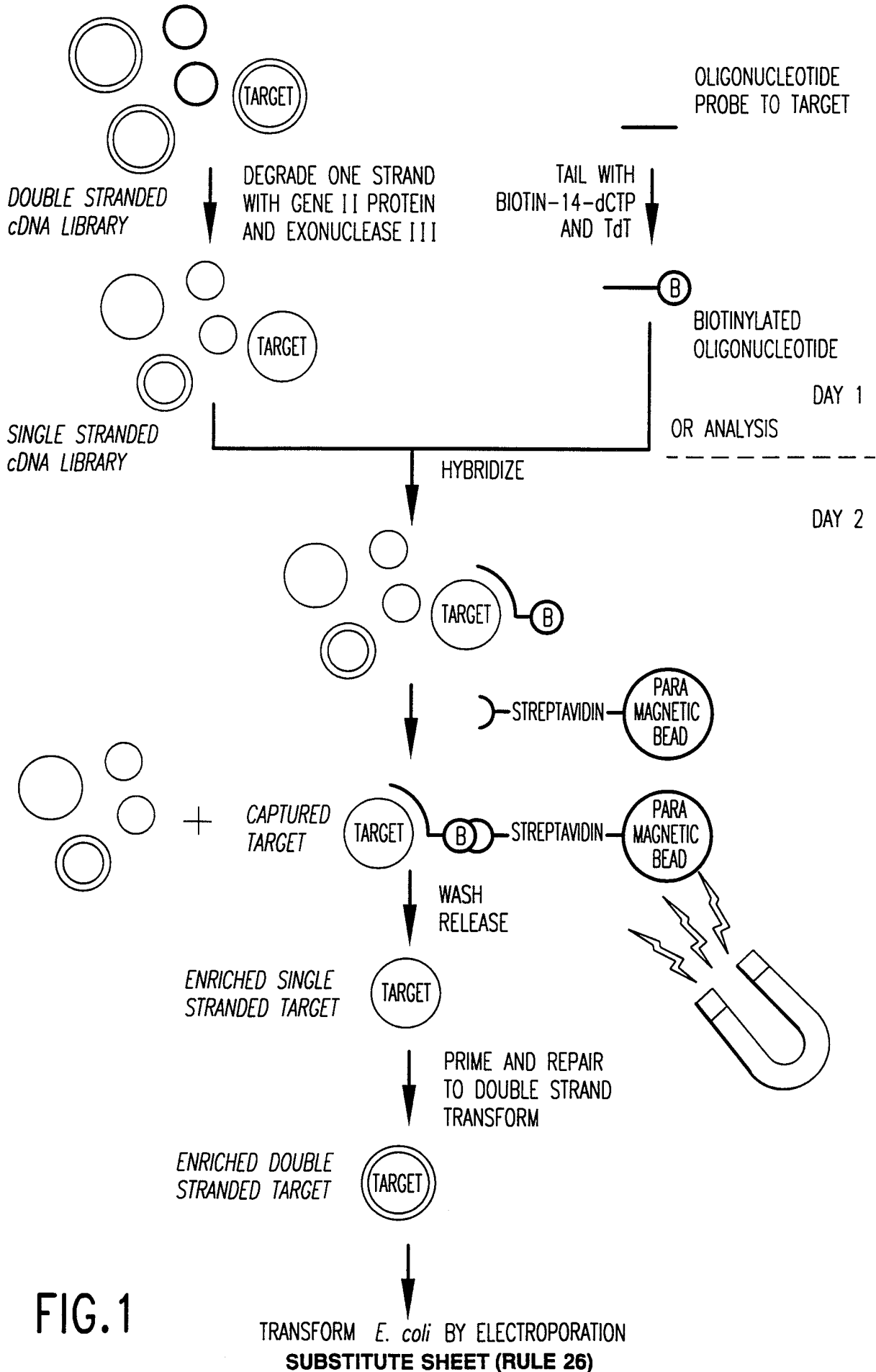


FIG. 1

TRANSFORM *E. coli* BY ELECTROPORATION  
SUBSTITUTE SHEET (RULE 26)

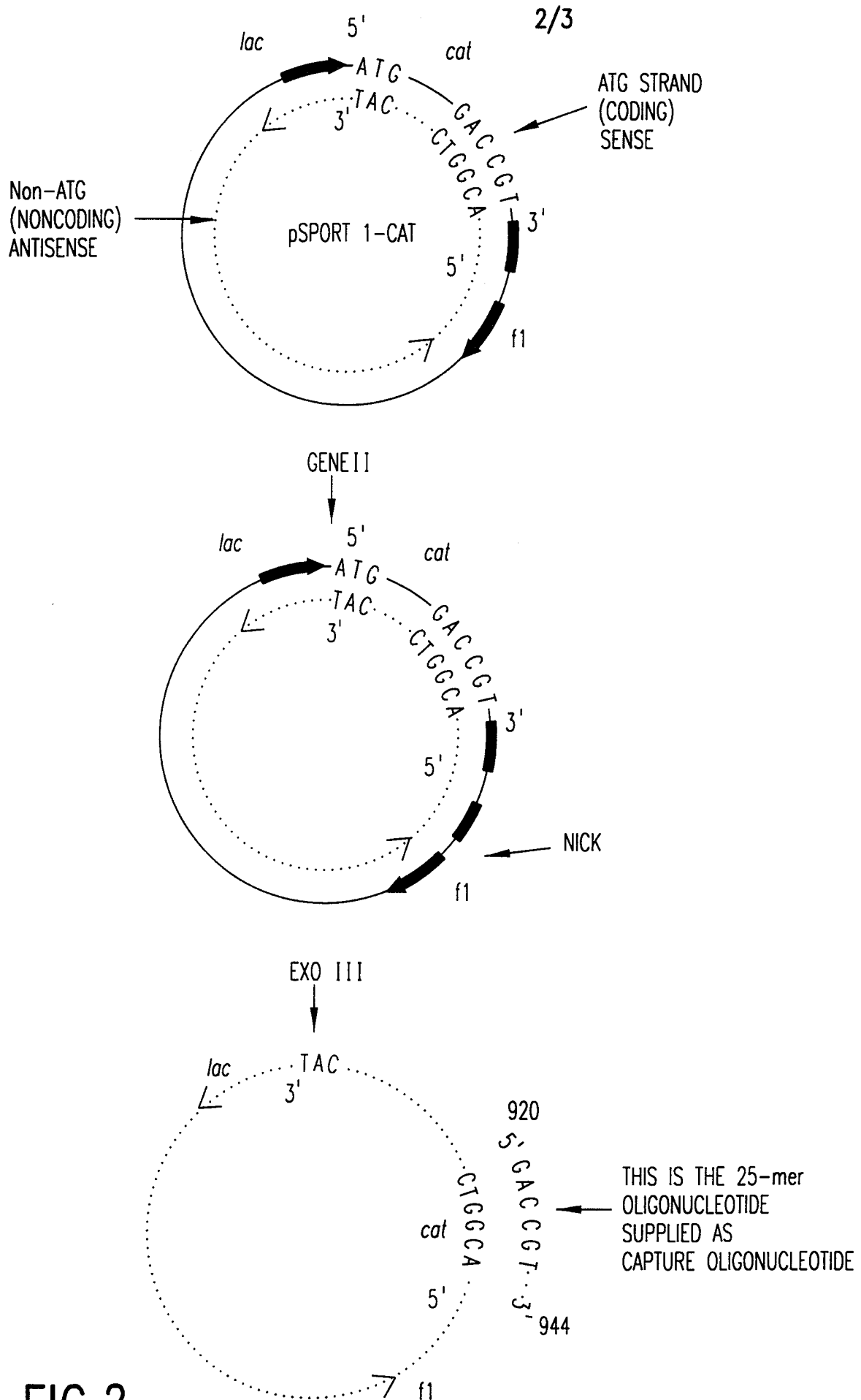


FIG.2

OVERVIEW OF GENE II-Exo III DIGESTION AND CAT OLIGONUCLEOTIDE BINDING

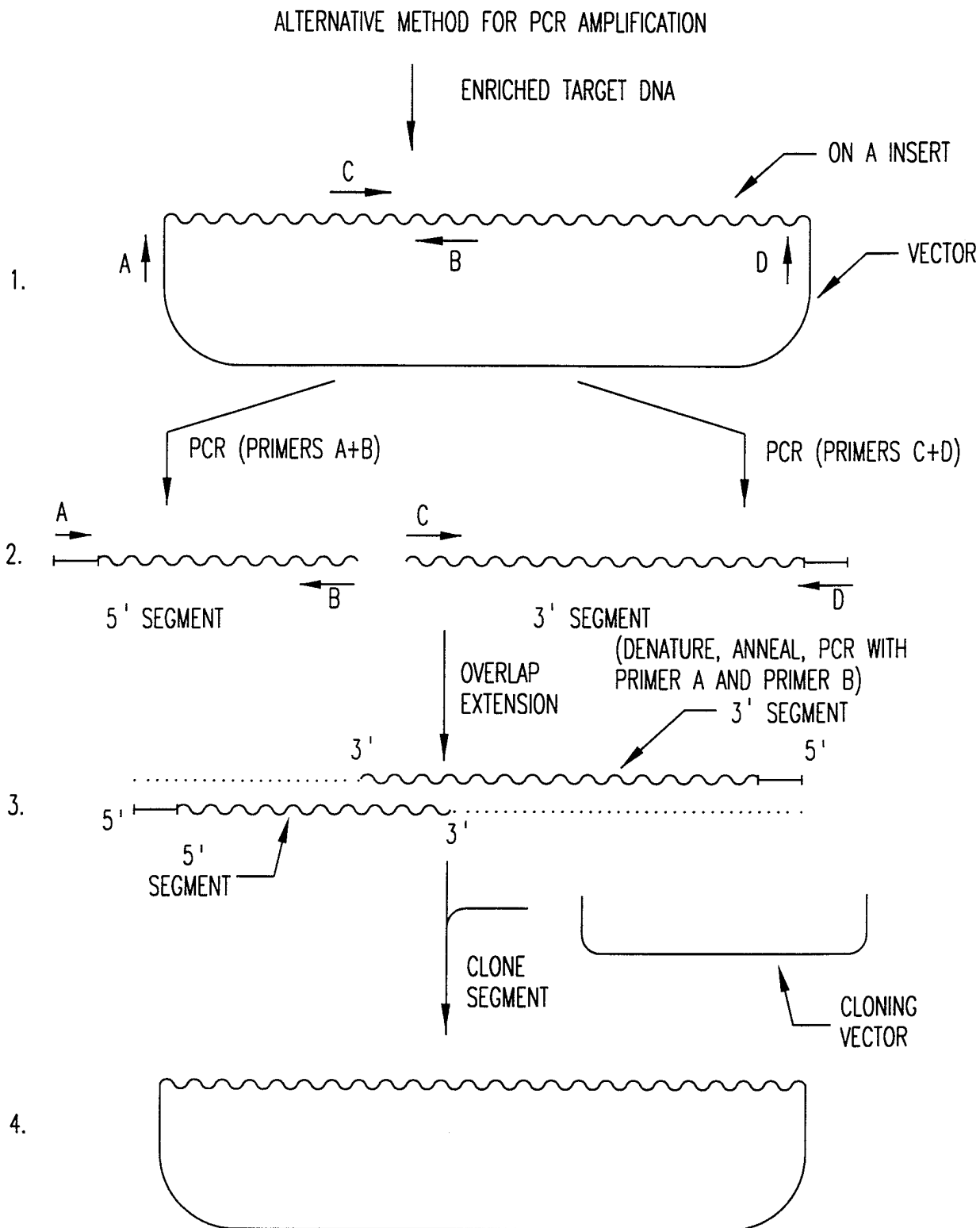


FIG.3

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/13043

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; G01N 33/48  
US CL :435/6; 436/94

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 436/94

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

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

APS, MEDLINE, CA, DERWENT, BIOSIS  
search terms: DNA, nucleic acid, denature, amino acid

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,165,925 A (LEONG) 24 November 1992, col. 8, lines 15-22.	1-40
A	US 5,350,672 A (OBERST et al.) 27 September 1994, col. 5, lines 8-12.	1-40
A	LIFE TECHNOLOGIES product catalogue and reference guide 1995-1996, page 19-10 and 19-12.	1-40

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

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 AUGUST 1998

Date of mailing of the international search report

24 SEP 1998

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