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(54) Title: METHODS, COMPOSITIONS AND KITS FOR PRODUCING NUCLEIC AND ACID SEQUENCES

(57) Abstract: The present invention relates to methods and compositions for the production of DNA and RNA, and in particular, double stranded DNA and double stranded RNA.

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METHODS, COMPOSITIONS AND KITS FOR PRODUCING NUCLEIC ACID SEQUENCES

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FIELD OF THE INVENTION

The present invention relates to methods and compositions for the production of DNA and RNA, and in particular, double stranded DNA and double stranded RNA.

BACKGROUND OF THE INVENTION

Double stranded RNA (dsRNA) mediated RNA interference (RNAi) technology has emerged as a powerful alternative tool for functional genomics. RNAi is now considered an outstanding method superior to conventional gene inhibition methods due to its speed, specificity and accuracy. RNAi is ubiquitous in nature and mechanistically conserved from fungi to human. Until recently, RNAi had been used as a gene silencing method mainly in model organisms such as *Caenorhabditis elegans* (nematode) and *Drosophila melanogaster* (fruit fly). These species are the two most popular organisms with which large scale genetic screening can be conducted. RNAi-based genomic analysis of gene function has already been reported in both species. What is needed is a more efficient way to produce a DNA sequence of interest and its corresponding dsRNA, preferably allowing use in a high-throughput format.

SUMMARY OF THE INVENTION

The present invention relates to the production of DNA sequences of interest and their corresponding double stranded RNA. The invention's methods are useful for the production of a DNA sequence of interest and double stranded RNA using, for example, a DNA sequence cloned in an expression vector (such as a plasmid) as well as using a DNA sequence which is not cloned. In some embodiments, the compositions, methods, and kits of the invention are also useful in determining whether a subject is at risk of developing a disease or condition depending on the DNA sequence of interest.

In one embodiment, the invention provides a method for producing a nucleic acid sequence, comprising: a) providing: i) a first double stranded DNA sequence of interest having a first strand and second strand, and comprising a first promoter; ii) a first primer comprising a first sequence complementary to the 3' end of at least a portion of the first promoter that is comprised on the first strand; iii) a second primer comprising a first sequence complementary to the 3' end of the second strand, and a second sequence complementary to at least a portion of the first promoter, wherein the second sequence is linked to the 5' end of the first sequence; and b) amplifying the first double stranded DNA sequence of interest in the presence of the first primer and the second primer to produce a first nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the at least a portion of the first promoter in a head to head orientation.

In another embodiment, the method further comprises: c) providing RNA polymerase that specifically binds to the first promoter; and d) contacting the first nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest. In an alternative embodiment, the method further comprises: c) providing a third primer complementary to the at least portion of the first promoter; and d) amplifying the first nucleic acid molecule produced in step b) in the presence of the third primer to produce a second nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the first promoter in a head to head orientation. In a more preferred embodiment, the method further comprises: e) providing RNA polymerase that specifically binds to the first promoter; f) contacting the second nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest.

While not intending to limit the DNA sequence to any particular sequence, in one embodiment, the DNA sequence of interest comprises a sequence chosen from one or more of cDNA, intron, exon, expression vector, and promoter. In another embodiment, the second strand of the double stranded DNA sequence of interest comprises at least a portion of a second promoter. In a further embodiment, the second promoter is different from the first promoter. In a yet more preferred embodiment, the first promoter comprises at least a portion of a promoter chosen from T7 promoter, T3 promoter, and

SP6 promoter, and the second promoter is chosen from T7 promoter, T3 promoter, and SP6 promoter.

Without intending to limit the invention to any particular promoter, in one embodiment, the first promoter comprises one or more of T7 promoter, T3 promoter, and SP6 promoter. Also without intending to limit any of the primers to a particular sequence, in one embodiment, the first strand of the double stranded DNA comprises a nucleotide sequence linked to the 3' end of the first promoter, and the first primer further comprises a second sequence complementary to the nucleotide sequence, wherein the second sequence is linked to the 3' end of the first sequence of the first primer. In an alternative embodiment, the first primer comprises a sequence complementary to one or more of T7 primer, such as SEQ ID NO:1, T3 promoter such as SEQ ID NO:8, and SP6 promoter, such as SEQ ID NO:9).

In another embodiment, the first sequence comprised on second primer is complementary to at least a portion of a promoter. Preferably, the promoter is chosen from one or more of T7 promoter, T3 promoter, and SP6 promoter. In an alternative embodiment, the second primer comprises one or more of T7-T3 primer such as SEQ ID NO:2 and 10, T7-SP6 such as SEQ ID NO:3 and 11, and T3-SP6 such as SEQ ID NO:12 and 13.

In one embodiment, the invention provides a method for producing a nucleic acid sequence, comprising: a) providing: i) a first double stranded DNA sequence of interest having a first strand and second strand; ii) a first primer comprising a first sequence complementary to the 3' end of the first strand, and a second sequence complementary to at least a portion of a promoter, wherein the second sequence is linked to the 5' end of the first sequence; iii) a second primer comprising a first sequence complementary to the 3' end of the second strand, and a second sequence complementary to at least a portion of the promoter, wherein the second sequence is linked to the 5' end of the first sequence; and b) amplifying the first double stranded DNA sequence of interest in the presence of the first primer and the second primer to produce a first nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the at least a portion of the promoter in a head to head orientation In another embodiment, the method further comprises: c) providing RNA polymerase that specifically binds to the promoter; and d) contacting the first nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA

sequence of interest. In an alternative embodiment, the method further comprises: c) providing a third primer complementary to the at least portion of the promoter; and d) amplifying the first nucleic acid molecule produced in step b) in the presence of the third primer to produce a second nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the promoter in a head to head orientation. In a more preferred embodiment, the method further comprises: e) providing RNA polymerase that specifically binds to the promoter; f) contacting the second nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest. While not intending to limit the invention to any DNA sequence, in one embodiment, the DNA sequence of interest comprises a sequence chosen from one or more of cDNA, intron, exon , expression vector, and promoter. Also without limiting the invention to any promoter, in one embodiment, the promoter comprises one or more of T7 promoter, T3 promoter, and SP6 promoter. Also without intending to limit the sequence of any of the primers, in one embodiment, the second sequence of the first primer comprises SEQ ID NO:6. In another embodiment, the second sequence of the second primer comprises SEQ ID NO:6. In yet a further embodiment, the third primer comprises a sequence complementary to one or more of T7 promoter such as SEQ ID NO:1 as illustrated in Figure 2, T3 promoter such as SEQ ID NO:8, and SP6 promoter such as SEQ ID NO:9.

The invention also provides a kit comprising: a) a first primer comprising a sequence complementary to at least a portion of a first promoter (such as T7, T3, and/or SP6; b) a second primer comprising a first sequence complementary to at least a portion of the first promoter, and a second sequence complementary to at least a portion of a second promoter (such as T7-T3 hybrid promoter (SEQ ID NO: 2 and 10), or T7-SP6 hybrid promoter (SEQ ID NO:3 and 11), and/or SP6-T3 hybrid promoter (SEQ ID NO:12 and 13)), wherein the first and second promoters are different; and c) instructions for producing a double stranded DNA sequence flanked by the first promoter in a head to head orientation or flanked by the second promoter in a head to head orientation, using the first primer and the second primer. In one embodiment, the kit further comprises: d) providing RNA polymerase that specifically binds to one or more of the first promoter and the second promoter; and e) instructions for producing a double stranded RNA sequence complementary to the DNA sequence using the RNA polymerase. While not limiting the invention to any particular promoter, in one embodiment, the first promoter

comprises a sequence chosen from one or more of T7 promoter (SEQ ID NO:1):, T3 promoter (SEQ ID NO:8), and SP6 promoter (SEQ ID NO:9). In another embodiment, the second promoter comprises a sequence chosen from one or more of T7 promoter (SEQ ID NO:1):, T3 promoter (SEQ ID NO:8), and SP6 promoter (SEQ ID NO:9). Also without limiting the primers to any particular sequence, in one embodiment, the second primer comprises a sequence chosen from one or more of T7-T3 primer (such as SEQ ID NO: 2 and 10), T7-SP6 primer (such as SEQ ID NO:3 and 11), and SP6-T3 hybrid primer (such as SEQ ID NO:12 and 13).

Also provided herein is a kit for producing a nucleic acid sequence, comprising: a) a first primer comprising a sequence complementary to at least a portion of a promoter; b) a second primer comprising a sequence complementary to at least a portion of the promoter; and c) instructions for producing a double stranded DNA sequence flanked by the first promoter in a head to head orientation using the first primer and the second primer. The first and second primers may be the same or different. In an alternative embodiment, the kit further comprises: d) providing RNA polymerase that specifically binds to the promoter; and e) instructions for producing a double stranded RNA sequence complementary to the DNA sequence using the RNA polymerase. Alternatively, the kit further comprises: d) providing a third primer comprising a sequence complementary to the promoter, wherein the third primer is different from the first and second primers; and e) instructions for producing a double stranded DNA sequence flanked by the third promoter in a head to head orientation using the third primer. More preferably, the kit further comprises: f) providing RNA polymerase that specifically binds to the promoter; and g) instructions for producing a double stranded RNA sequence complementary to the DNA sequence using the RNA polymerase.

While not limiting the promoter to any particular sequence, the promoter comprises a sequence chosen from one or more of T7 promoter (SEQ ID NO:1):, T3 promoter (SEQ ID NO:8), and SP6 promoter (SEQ ID NO:9).

In one embodiment, the invention provides a method for producing a DNA sequence of interest flanked by a T7 promoter in a head to head orientation, comprising: a) providing: i) a first sequence comprising a DNA sequence of interest flanked by 1) a T7 promoter, and 2) a hybrid promoter selected from the group consisting of T7-T3 and T7-SP6; ii) a first primer complementary to the T7 promoter, the first primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); iii) a second primer

complementary to the hybrid promoter, the second primer comprising 5'-TAATACGACTCACTATAGGGATTAACCCTCACTAAAGGGA-3' (SEQ ID NO:2), or 5'-TAATACGACTCACTATAGGGTATTTAGGTGACACTATAG-3' (SEQ ID NO:3); and b) amplifying the DNA sequence of interest in the presence of the first primer and the second primer such that a second sequence is produced, wherein the second sequence comprises the DNA sequence of interest flanked by a T7 promoter in a head to head orientation. The DNA produced by the invention's methods may be single stranded or double stranded.

In another embodiment, the invention provides a method for producing double stranded RNA that is complementary to a DNA sequence of interest, comprising the steps of Claim 1, and further comprising: c) providing T7 RNA polymerase; d) contacting the second sequence with the T7 RNA polymerase such that double stranded RNA that is complementary to the DNA sequence of interest in produced.

In a further embodiment, the invention provides a method for producing a DNA sequence of interest flanked by a T7 promoter in a head to head orientation, comprising: a) providing: i) a first sequence comprising a DNA sequence of interest; ii) a first primer comprising a sequence complementary to the 5' end of the DNA sequence of interest operably linked to the hexanucleotide 5'-ataggg-3' (SEQ ID NO:6), wherein the hexanucleotide is at the 3' end of the first primer; iii) a second primer comprising a sequence complementary to the 3' end of the DNA sequence of interest operably linked to the hexanucleotide 5'-ataggg-3' (SEQ ID NO:6), wherein the hexanucleotide is at the 5' end of the second primer; and iv) a third primer complementary to a T7 promoter, the third primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); b) amplifying the DNA sequence of interest in the presence of the first primer and the second primer such that a second sequence is produced, wherein the second sequence comprises the DNA sequence of interest flanked by 5'-tatccc-3' (SEQ ID NO:5) at the 5' end and by 5'-gggata-3' (SEQ ID NO:7) at the 3' end; c) amplifying the DNA sequence of interest in the second sequence in the presence of the third primer such that a third sequence is produced, wherein the third sequence comprises the DNA sequence of interest flanked by a T7 promoter in a head to head orientation.

In yet another embodiment, the invention provides a method for producing double stranded RNA that is complementary to a DNA sequence of interest, comprising the steps of Claim 3, and further comprising: d) providing T7 RNA polymerase; e) contacting the

second sequence with the T7 RNA polymerase such that double stranded RNA that is complementary to the DNA sequence of interest in produced.

In a further embodiment, the invention provides a kit for producing a DNA sequence of interest flanked by T7 polymerase promoter in a head to head orientation, comprising: a) a first primer complementary to a T7 promoter, the first primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); b) a second primer complementary to a T7-T3 hybrid promoter, the second primer comprising 5'-TAATACGACTCACTATAGGGATTAACCCTCACTAAAGGGA-3' (SEQ ID NO: 2); c) instructions for producing the DNA sequence of interest using the first primer and the second primer.

In an alternative embodiment, the invention provides a kit for producing a DNA sequence of interest flanked by T7 polymerase promoter in a head to head orientation, comprising: a) a first primer complementary to a T7 promoter, the first primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); b) a second primer complementary to a T7-SP6 hybrid promoter, the second primer comprising 5'-TAATACGACTCACTATAGGGTATTTAGGTGACACTATAG-3' (SEQ ID NO:3); and c) instructions for producing the DNA sequence of interest using the first primer and the second primer.

In an additional embodiment, the invention provides a kit for producing a DNA sequence of interest flanked by T7 polymerase promoter in a head to head orientation, comprising: a) a first primer comprising hexanucleotide 5'-ataggg-3' (SEQ ID NO:6) at the 3' end of the first primer; b) a second primer comprising hexanucleotide 5'-ataggg-3' (SEQ ID NO:6) at the 5' end of the second primer; c) a third primer complementary to a T7 promoter, the third primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); and d) instructions for producing the DNA sequence of interest using the first primer, the second primer, and the third primer.

DEFINITIONS

To facilitate understanding of the invention, a number of terms are defined below.

The terms "nucleic acid sequence" and "nucleotide sequence" as used herein refer to two or more nucleotides which are covalently linked to each other. Included within this definition are oligonucleotides, polynucleotide, and fragments or portions thereof, deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") sequences of genomic or

synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

As used herein the term "portion" when made in reference to a nucleic acid sequence refers to a fragment of that sequence. The fragment may range in size from an exemplary 1, 5, 10, 20, 50, and/or 100 contiguous nucleotide residues to the entire nucleic acid sequence minus one nucleic acid residue. Thus, a nucleic acid sequence comprising "at least a portion of" a nucleotide sequence comprises from one (1) nucleotide residue of the nucleotide sequence to the entire nucleotide sequence.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides or polynucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotide or polynucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide or polynucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements that direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

The term "5' end" as used herein in reference to a nucleic acid sequence refers to a portion of the nucleic acid sequence from one nucleotide at the terminal 5' end to about the center nucleotide of the nucleic acid sequence. Thus, the term "5' end" includes the terminal single nucleotide a the 5' end, as well as portions located anywhere from the terminal single nucleotide to about the center nucleotide of the nucleic acid sequence. Similarly, the term "3' end" as used herein in reference to a nucleic acid sequence refers to a portion of the nucleic acid sequence from one nucleotide at the terminal 3' end to about the center nucleotide of the nucleic acid sequence. Thus, the term "3' end" includes the terminal single nucleotide a the 3' end, as well as portions

located anywhere from the terminal single nucleotide to about the center nucleotide of the nucleic acid sequence.

As used herein, the terms "complementary" or "complementarity" are used in reference to nucleotide sequences related by the base-pairing rules. For example, the sequence 5'-AGT-3' is complementary to the sequence 5'-ACT-3'. Complementarity can be "partial" or "total." "Partial" complementarity is where one or more nucleic acid bases is not matched according to the base pairing rules. "Total" or "complete" complementarity between nucleic acids is where each and every nucleic acid base is matched with another base under the base pairing rules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands.

The term "hybridization" as used herein includes "any process by which a strand of nucleic acid joins with a complementary strand through base pairing." (Coombs J (1994) *Dictionary of Biotechnology*, Stockton Press, New York NY). Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementarity between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids.

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*see e.g.,* Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* (1985)). Other references include more sophisticated computations which take structural as well as sequence characteristics into account for the calculation of T_m.

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. Those skilled in the art will recognize that "stringency" conditions may be altered by varying the parameters just described either individually or in concert. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high

frequency of complementary base sequences (e.g., hybridization under "high stringency" conditions may occur between homologs with about 85-100% identity, preferably about 70-100% identity). With medium stringency conditions, nucleic acid base pairing will occur between nucleic acids with an intermediate frequency of complementary base sequences (e.g., hybridization under "medium stringency" conditions may occur between homologs with about 50-70% identity). Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

"High stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH2PO4-H2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed. In another embodiment, high stringency conditions comprise conditions equivalent to binding or hybridization at 68°C in a solution containing 5X SSPE, 1% SDS, 5X Denhardt's reagent and 100 μg/ml denatured salmon sperm DNA followed by washing in a solution containing 0.1X SSPE, and 0.1% SDS at 68°C when a probe of about 100 to about 1000 nucleotides in length is employed.

"Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH_2PO_4-H_2O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 ìg/ml denatured salmon sperm DNA followed by washing in a solution comprising 1.0X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

"Low stringency conditions" comprise conditions equivalent to binding or hybridization at 42°C in a solution of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $NaH_2PO_4H_2O$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent (50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharamcia), 5 g BSA (Fraction V; Sigma)) and 100 Fg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

The term "equivalent" when made in reference to a hybridization condition as it relates to a hybridization condition of interest means that the hybridization condition and the hybridization condition of interest result in hybridization of nucleic acid sequences which have the same range of percent (%) homology. For example, if a hybridization condition of interest results in hybridization of a first nucleic acid sequence with other nucleic acid sequences that have from 50% to 70% homology to the first nucleic acid sequence, then another hybridization condition is said to be equivalent to the hybridization condition of interest if this other hybridization condition also results in hybridization of the first nucleic acid sequence with the other nucleic acid sequences that have from 50% to 70% homology to the first nucleic acid sequence.

When used in reference to nucleic acid hybridization the art knows well that numerous equivalent conditions may be employed to comprise either low or high stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency hybridization different from, but equivalent to, the above-listed conditions.

Those skilled in the art know that whereas higher stringencies may be preferred to reduce or eliminate non-specific binding between the nucleotide sequence of interest and other nucleic acid sequences, lower stringencies may be preferred to detect and/or hybridize to a larger number of nucleic acid sequences having different homologies to the nucleotide sequence of interest.

As used herein the term "hybridization complex" refers to a complex formed between two nucleic acid sequences by virtue of the formation of hydrogen bounds between complementary G and C bases and between complementary A and T bases; these hydrogen bonds may be further stabilized by base stacking interactions. The two complementary nucleic acid sequences hydrogen bond in an antiparallel configuration. A hybridization complex may be formed in solution (*e.g.*, C_0t or R_0t analysis) or between one nucleic acid sequence present in solution and another nucleic acid sequence immobilized to a solid support (*e.g.*, a nylon membrane or a nitrocellulose filter as

employed in Southern and Northern blotting, dot blotting or a glass slide as employed in *in situ* hybridization, including FISH (fluorescent *in situ* hybridization).

The terms "link," "join," "fuse," "splice," and "ligate" when in reference to two or more nucleic acid sequences, two or more polypeptide sequences, and/or mixtures of nucleic acid sequences and polypeptide sequences, mean that the sequences are connected via covalent and/or non-covalent bonds, preferably via covalent bonds. The term "link" includes connecting the sequence in the absence and/or presence of intervening sequences and/or molecules (such as carbohydrate, saccharide, polysaccharide, lipid, *etc.*).

The term "operably linked" when in reference to the relationship between nucleic acid sequences and/or amino acid sequences refers to linking the sequences such that they perform their intended function. For example, operably linking a promoter sequence to a nucleotide sequence of interest refers to linking the promoter sequence and the nucleotide sequence of interest in a manner such that the promoter sequence is capable of directing the transcription of the nucleotide sequence of interest and/or the synthesis of a polypeptide encoded by the nucleotide sequence of interest. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer is selected such that it is sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. Suitable lengths of the primers may be empirically determined and depend on factors such as temperature, source of primer and the use of the method. In one embodiment, the primers may be from 3 to 100, preferably from 3 to 50, more preferably from 3 to 25 nucleotide bases in length. In another embodiment, the primer is from 20 to 50 nucleotide bases in length.

As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

The terms "peptide of interest," "nucleotide sequence of interest," "DNA sequence of interest," and "molecule of interest" refer to any peptide sequence, nucleotide sequence, DNA sequence, and molecule, respectively, the manipulation of which may be deemed desirable for any reason, by one of ordinary skill in the art. The term "antisense DNA sequence" as used herein refers to a deoxyribonucleotide sequence whose sequence of deoxyribonucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of deoxyribonucleotide residues in a sense strand of a DNA duplex. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA." Sense mRNA generally is ultimately translated into a polypeptide. Thus an "antisense DNA sequence" is a sequence which has the same sequence as the non-coding strand in a DNA duplex, and which encodes an "antisense RNA," *i.e.*, a ribonucleotide sequence whose sequence is complementary to a "sense mRNA" sequence. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand. Antisense RNA may be produced by any method, including synthesis by splicing an antisense DNA sequence to a promoter which permits the synthesis of antisense RNA. The transcribed antisense RNA strand combines with natural mRNA produced by the cell to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation, or promote its degradation. Thus, antisense DNA sequences are useful in, for example, inhibiting the activity of a protein which is produced by a pathogenic gene or which is present in the genome of a pathogenic organism. Alternatively, antisense DNA sequences may be used to inhibit a cellular gene whose expression is deregulated (*e.g.*, an oncogene). Methods of generating and using antisense DNA sequences are known in the art (see for example, patent EP 140 308).

The term "Southern blot" refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size, followed by transfer and immobilization of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane.

The immobilized DNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (J. Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58).

The term "Northern blot" as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled oligo-deoxyribonucleotide probe or DNA probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (J. Sambrook, J. *et al.* (1989) *supra*, pp 7.39-7.52).

The term "reverse Northern blot" as used herein refers to the analysis of DNA by electrophoresis of DNA on agarose gels to fractionate the DNA on the basis of size followed by transfer of the fractionated DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled oligo-ribonucleotide probe or RNA probe to detect DNA species complementary to the ribo probe used.

The term "Western blot" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an antigen of interest. The binding of the antibodies may be detected by various methods, including the use of radiolabeled antibodies.

As used herein, the terms "vector" and "vehicle" are used interchangeably in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. Vecrtos are exemplified by, but not limited to, plasmids, linear DNA, encapsidated virus, *etc.*

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences

necessary for the expression of the operably linked coding sequence in a particular host organism. Expression vectors are exemplified by, but not limited to, plasmid, phagemid, shuttle vector, cosmid, and virus. Nucleic acid sequences used for expression in prokaryotes include a promoter, optionally an operator sequence, a ribosome binding site and possibly other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

Vectors may be introduced into cells using techniques well known in the art. The term "introducing" a nucleic acid sequence into a cell refers to the introduction of the nucleic acid sequence into a target cell to produce a transformed cell. Methods of introducing nucleic acid sequences into cells are well known in the art. For example, where the nucleic acid sequence is a plasmid or naked piece of linear DNA, the sequence may be "transfected" into the cell using, for example, calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, and biolistics. Alternatively, where the nucleic acid sequence is encapsidated into a viral particle, the sequence may be introduced into a cell by "infecting" the cell with the virus. In a preferred embodiment, the vectors of the invention are encapsidated into viral particles and used to infect cells to bring about cell transformation.

Transformation of a cell may be stable or transient. The terms "transient transformation" and "transiently transformed" refer to the introduction of one or more nucleotide sequences of interest into a cell in the absence of integration of the nucleotide sequence of interest into the host cell's genome. Transient transformation may be detected by, for example, enzyme-linked immunosorbent assay (ELISA) which detects the presence of a polypeptide encoded by one or more of the nucleotide sequences of interest. Alternatively, transient transformation may be detected by detecting the activity of the protein (*e.g.*, β-glucuronidase) encoded by the nucleotide sequence of interest. The term "transient transformant" refer to a cell which has transiently incorporated one or more nucleotide sequences of interest. Transient transformation with the invention's vectors may be desirable in, for example, cell biology or cell cycle investigations which require efficient gene transfer.

In contrast, the terms "stable transformation" and "stably transformed" refer to the introduction and integration of one or more nucleotide sequence of interest into the genome of a cell. Thus, a "stable transformant" is distinguished from a transient

transformant in that, whereas genomic DNA from the stable transformant contains one or more nucleotide sequences of interest, genomic DNA from the transient transformant does not contain the nucleotide sequence of interest. Stable transformation of a cell may be detected by Southern blot hybridization of genomic DNA of the cell with nucleic acid sequences which are capable of binding to one or more of the nucleotide sequences of interest. Alternatively, stable transformation of a cell may also be detected by the polymerase chain reaction of genomic DNA of the cell to amplify the nucleotide sequence of interest. In a preferred embodiment, transformation is stable.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" includes both singular and plural references unless the content clearly dictates otherwise.

As used herein, the term "or" when used in the expression "A or B," where A and B refer to a composition, disease, product, *etc.*, means one, or the other, or both.

The term "on" when in reference to the location of a first article with respect to a second article means that the first article is on top and/or into the second article, including, for example, where the first article permeates into the second article after initially being placed on it.

As used herein, the term "comprising" when placed before the recitation of steps in a method means that the method encompasses one or more steps that are additional to those expressly recited, and that the additional one or more steps may be performed before, between, and/or after the recited steps. For example, a method comprising steps a, b, and c encompasses a method of steps a, b, x, and c, a method of steps a, b, c, and x, as well as a method of steps x, a, b, and c. Furthermore, the term "comprising" when placed before the recitation of steps in a method does not (although it may) require sequential performance of the listed steps, unless the content clearly dictates otherwise. For example, a method comprising steps a, b, and c encompasses, for example, a method of performing steps in the order of steps a, c, and b, the order of steps c, b, and a, and the order of steps c, a, and b, *etc.*

Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth as used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters in the specification and claims are approximations that may vary depending upon the

desired properties sought to be obtained by the present invention. At the very least, and without limiting the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters describing the broad scope of the invention are approximation, the numerical values in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains standard deviations that necessarily result from the errors found in the numerical value's testing measurements.

The term "not" when preceding, and made in reference to, any particularly named molecule (such as T7, T3, SP6, mRNA, DNA, *etc.*) or phenomenon (such as biological activity, biochemical activity, etc.) means that only the particularly named molecule or phenomenon is excluded.

The term "altering" and grammatical equivalents as used herein in reference to the level of any substance and/or phenomenon refers to an increase and/or decrease in the quantity of the substance and/or phenomenon, regardless of whether the quantity is determined objectively, and/or subjectively.

The term "increase," "elevate," "raise," and grammatical equivalents when in reference to the level of a substance and/or phenomenon in a first sample relative to a second sample, mean that the quantity of the substance and/or phenomenon in the first sample is higher than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the increase may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, clarity of vision, *etc.*. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 10% greater than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% greater than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% greater than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% greater than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or

phenomenon in the first sample is at least 90% greater than the quantity of the same substance and/or phenomenon in a second sample.

The terms "reduce," "inhibit," "diminish," "suppress," "decrease," and grammatical equivalents when in reference to the level of a substance and/or phenomenon in a first sample relative to a second sample, mean that the quantity of substance and/or phenomenon in the first sample is lower than in the second sample by any amount that is statistically significant using any art-accepted statistical method of analysis. In one embodiment, the reduction may be determined subjectively, for example when a patient refers to their subjective perception of disease symptoms, such as pain, clarity of vision, *etc.*. In another embodiment, the quantity of substance and/or phenomenon in the first sample is at least 10% lower than the quantity of the same substance and/or phenomenon in a second sample. In another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 25% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 50% lower than the quantity of the same substance and/or phenomenon in a second sample. In a further embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 75% lower than the quantity of the same substance and/or phenomenon in a second sample. In yet another embodiment, the quantity of the substance and/or phenomenon in the first sample is at least 90% lower than the quantity of the same substance and/or phenomenon in a second sample.

Reference herein to any specifically named protein (such as "RNA polymerase," *etc.*) refers to a polypeptide having at least one of the biological activities of the specifically named protein, wherein the biological activity is detectable by any method. Also, reference herein to any specifically named protein (such as "RNA polymerase," *etc.*) includes within its scope fragments, fusion proteins, and variants of the specifically named protein. The term "fragment" when in reference to a protein refers to a portion of that protein that may range in size from four (4) contiguous amino acid residues to the entire amino acid sequence minus one amino acid residue. Thus, a polypeptide sequence comprising "at least a portion of an amino acid sequence" comprises from four (4) contiguous amino acid residues of the amino acid sequence to the entire amino acid sequence. The term A "variant" of a protein as used herein is defined as an amino acid sequence which differs by insertion, deletion, and/or conservative substitution of one or

more amino acids from the protein. The term "conservative substitution" of an amino acid refers to the replacement of that amino acid with another amino acid which has a similar hydrophobicity, polarity, and/or structure. For example, the following aliphatic amino acids with neutral side chains may be conservatively substituted one for the other: glycine, alanine, valine, leucine, isoleucine, serine, and threonine. Aromatic amino acids with neutral side chains which may be conservatively substituted one for the other include phenylalanine, tyrosine, and tryptophan. Cysteine and methionine are sulphur-containing amino acids which may be conservatively substituted one for the other. Also, asparagine may be conservatively substituted for glutamine, and *vice versa*, since both amino acids are amides of dicarboxylic amino acids. In addition, aspartic acid (aspartate) my be conservatively substituted for glutamic acid (glutamate) as both are acidic, charged (hydrophilic) amino acids. Also, lysine, arginine, and histidine my be conservatively substituted one for the other since each is a basic, charged (hydrophilic) amino acid. Guidance in determining which and how many amino acid residues may be substituted, inserted or deleted without abolishing biological and/or immunological activity may be found using computer programs well known in the art, for example, DNAStar™ software. In one embodiment, the sequence of the variant has at least 95% identity with the sequence of the protein in issue. In another embodiment, the sequence of the variant has at least 90% identity with the sequence of the protein in issue. In yet another embodiment, the sequence of the variant has at least 85% identity with the sequence of the protein in issue. In a further embodiment, the sequence of the variant has at least 80% identity with the sequence of the protein in issue. In yet another embodiment, the sequence of the variant has at least 75% identity with the sequence of the protein in issue. In another embodiment, the sequence of the variant has at least 70% identity with the sequence of the protein in issue. In another embodiment, the sequence of the variant has at least 65% identity with the sequence of the protein in issue.

Reference herein to any specifically named nucleotide sequence (such as a DNA sequence, RNA sequence, *etc.*) includes within its scope "portions," homologs, and sequences that hybridize under high and/or medium stringent conditions to the specifically named nucleotide sequence.

The term "homolog" of a specifically named nucleotide sequence refers to an oligonucleotide sequence which exhibits greater than or equal to 50% identity to the specifically named nucleotide sequence. Alternatively, a homolog of a specifically

named nucleotide sequence is defined as an oligonucleotide sequence which has at least 95% identity with the sequence of the nucleotide sequence in issue. In another embodiment, the sequence of the homolog has at least 90% identity with the sequence of the nucleotide sequence in issue. In yet another embodiment, the sequence of the homolog has at least 85% identity with the sequence of the nucleotide sequence in issue. In a further embodiment, the sequence of the homolog has at least 80% identity with the sequence of the nucleotide sequence in issue. In yet another embodiment, the sequence of the homolog has at least 75% identity with the sequence of the nucleotide sequence in issue. In another embodiment, the sequence of the homolog has at least 70% identity with the sequence of the nucleotide sequence in issue. In another embodiment, the sequence of the homolog has at least 65% identity with the sequence of the nucleotide sequence in issue.

The term "naturally occurring" as used herein when applied to an object (such as cell, *etc.*) and/or chemical (such as amino acid, amino acid sequence, nucleic acid, nucleic acid sequence, codon, *etc.*) means that the object and/or compound can be found in nature. For example, a naturally occurring polypeptide sequence refers to a polypeptide sequence that is present in an organism (including viruses) that can be isolated from a source in nature, wherein the polypeptide sequence has not been intentionally modified by man in the laboratory.

The terms nucleotide sequence "comprising a particular nucleic acid sequence" and protein "comprising a particular amino acid sequence" and equivalents of these terms, refer to any nucleotide sequence of interest and to any protein of interest that contains the particularly named nucleic acid sequence and the particularly named amino acid sequence, respectively. The invention does not limit the source (*e.g.*, cell type, tissue, animal, *etc.*), nature (*e.g.*, synthetic, recombinant, purified from cell extract, *etc.*), and/or sequence of the nucleotide sequence of interest and/or protein of interest. In one embodiment, the nucleotide sequence of interest and protein of interest include coding sequences of structural genes (*e.g.*, probe genes, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*).

Exemplary "probe" genes sequences (*i.e.*, sequence useful in the detection, identification and isolation of particular polypeptide sequence) encode ligand-binding systems useful for the isolation of polypeptides such as the staphylococcal protein A and its derivative ZZ (which binds to human polyclonal IgG), histidine tails (which bind to

Ni^{2+}), biotin (which binds to streptavidin), maltose-binding protein (MBP) (which binds to amylose), glutathione S-transferase (which binds to glutathione), *etc.* Exemplary "reporter" gene sequences (*i.e.* sequences that encodes a molecule such as RNA, polypeptide, *etc.*, that is detectable in enzyme-based histochemical assays, fluorescent, radioactive, and luminescent systems, *etc.*) include green fluorescent protein gene, *E. coli* β-galactosidase gene, human placental alkaline phosphatase gene, and chloramphenicol acetyltransferase gene.

The term "chosen from A, B and C" means selecting one or more of A, B, and C.

A "composition comprising a particular polynucleotide sequence" as used herein refers broadly to any composition containing the recited polynucleotide sequence. The composition may comprise an aqueous solution containing, for example, salts (e.g., NaCl), detergents (e.g., SDS), and other components (e.g., Denhardt's solution, dry milk, salmon sperm DNA, etc.).

DESCRIPTION OF THE FIGURES

Figure 1 shows an exemplary method which illustrates preparation of transcription templates from a DNA sequence cloned in a plasmid vector.

Figure 2 shows an exemplary method which illustrates preparation of transcription templates from a DNA sequence without a cloning procedure.

Figure 3 shows a Western blot of proteins from *Drosophila* S2 cells.

Figure 4 shows a graph with *Drosophila* % mRNA level versus dsRNA treatment.

GENERAL DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods for producing DNA sequences of interest and double stranded RNA that is complementary to the DNA sequence of interest. In particular, the present invention provides methods and compositions useful for producing a DNA sequence of interest as well as double stranded RNA ("dsRNA") with or without having previously cloned the DNA sequence of interest. More specifically, in one embodiment, the present invention is useful for producing dsRNA by T7 RNA polymerase from DNA templates that have been directly amplified from genomic DNA, cDNA, and/or expression vector templates.

Some of the advantages of the present invention include, but are not limited to, the following. First, the invention's methods do not require, but may include, a cloning

procedure to obtain T7 transcription templates. Second, the invention's methods may be carried out without extraction and precipitation of the genetic material. Third, the invention's methods may be performed in a single container.

The present invention provides examples (see below) for production of DNA and/or dsRNA, which can then be used, for example, for transfection into cells such as (without limitation) cultured *Drosophila*. Alternatively, DNA and/or dsRNA which is produced in accordance with the invention's methods may be injected into cells, tissues, and organisms, such as (without limitation) *C. elegans* and *Drosophila* embryos. (Ramet et al., Nature, 416: 644-648 (2002)). Additional uses for the present invention include, but are not limited to, dsRNA kits for producing DNA sequences of interest or double stranded RNA. These kits may be used for RNA interference ("RNAi") in any organism, such as *Drosophila* and *C. elegans* or an automatic procedure for high-throughput RNAi screening (Hannon, Nature, 418: 244-251 (2002)).

The present invention also finds use in mammals. As used herein, the "mammal" includes a rodent, primate (including simian and human) ovine, bovine, ruminant, lagomorph, porcine, caprine, equine, canine, feline, ave, *etc.* Preferred non-human animals are selected from the order Rodentia, such as mouse and rat. Recently, RNAi has been successfully performed in mammalian cells by using very short dsRNA called small interfering RNA (siRNA) (Elbashir *et al.*, Nature, 411: 494-498 (2001)). It has long been held that RNAi was not efficacious in mammalian cells because treatment with long dsRNA caused general cytotoxicity as well as specific gene silencing. However, very short (usually, about 21-nucleotides depending on individual species) dsRNA does not result in such general cytotoxicity toward mammalian cells while retaining the capacity to transiently induce RNAi. Stable introduction of RNAi into mammalian cells was also achieved by using a method in which plasmid vectors that expresses siRNA is transfected into the cells (Brummelkamp, *et al.*, Science 296: 550-553. (2002); Tuschl, Nat. Biotechnol., 20:446-448 (2002)). With RNAi technology applicable to mammalian cells, high-throughput functional genomic analysis (initially imaginable only in model organisms like *C. elegans* and *Drosophila*) is now open to mammalian cells. (Maeda *et al.*, Curr. Biol., 11:171-176 (2001)). The present invention provides the methods needed to accomplish high-throughput genomic analyses.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the production of DNA sequences of interest and their corresponding double stranded RNA. The invention's methods are useful for the production of a DNA sequence of interest and double stranded RNA using a DNA sequence cloned in a plasmid vector as well as using a DNA sequence which is not cloned. In some embodiments, the compositions, methods, and kits of the invention are also useful in determining whether a subject is at risk of developing a disease or condition that depends on the DNA sequence of interest. Such a determination may be made by using, for example, double stranded RNA (dsRNA) mediated RNA interference (RNAi) technology (see, Example 6 herein, (Elbashir *et al.*, (2001), Ramet et al., (2002), and Hannon (2002)). To facilitate understanding of the invention, the invention is further described under (A) Production of a DNA Sequence of Interest and double stranded RNA Using a DNA Sequence Linked to Additional Sequences, (B) Production of a DNA Sequence of Interest and Double Stranded RNA From a DNA Sequence That Is Not Linked to Additional Sequences, and (C) Kits for Producing a DNA Sequence of Interest And Double Stranded RNA.

A. Production of a DNA Sequence of Interest and double stranded RNA Using a DNA Sequence Linked to Additional Sequences

In one embodiment, the invention provides a method for producing a nucleic acid sequence, comprising: a) providing: i) a first double stranded DNA sequence of interest having a first strand and second strand, and comprising a first promoter; ii) a first primer comprising (for example at its 5' end) a first sequence complementary to the 3' end of at least a portion of the first promoter that is comprised on the first strand; iii) a second primer comprising a first sequence complementary to the 3' end of the second strand, and a second sequence complementary to at least a portion of the first promoter, wherein the second sequence is linked to the 5' end of the first sequence; and b) amplifying the first double stranded DNA sequence of interest in the presence of the first primer and the second primer to produce a first nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the at least a portion of the first promoter in a head to head orientation. This method is exemplified in Figure 1. The resulting double stranded DNA sequence may be flanked by a portion or the promoter, or by the full length promoter.

The term "promoter," "promoter element," or "promoter sequence" as used herein, refers to a DNA sequence which when ligated to a nucleotide sequence of interest is capable of controlling the transcription of the nucleotide sequence of interest into mRNA. A promoter is typically, though not necessarily, located 5' (*i.e.*, upstream) of a nucleotide sequence of interest whose transcription into mRNA it controls, and provides a site for specific binding by RNA polymerase and other transcription factors for initiation of transcription. The term "promoter" encompasses a single promoter sequence as well as to a plurality (*i.e.*, one or more) of promoter sequences which are operably linked to each other and to at least one DNA sequence of interest. For example, one of skill in the art knows that it may be desirable to use a double promoter sequence (*i.e.*, a DNA sequence containing two promoter sequences) or a triple promoter sequence (*i.e.*, a DNA sequence containing three promoter sequences) to control expression of a DNA sequence of interest. Double promoters are exemplified, but not limited to, T7-T3 (such as SEQ ID NO:2 5'-TAATACGACTCACTATAGGGATTAACCCTCACTAAAGGGA-3'), T3-T7 (such as SEQ ID NO:10 5'-attaaccctcactaaagggaTAATACGACTCACTATAGGG-3'), T7-SP6 (such as SEQ ID NO:3 5'-TAATACGACTCACTATAGGGT ATTTAGGTGACACTATAG-3'), SP6-T7 (such as SEQ ID NO:11 5'-tatttaggtgacactatagTAATACGACTCACTATAGGG-3'), SP6-T3 (such as SEQ ID NO:12 5'-tatttaggtgacactatagattaaccctcactaaaggga-3'), T3-SP6 (such as SEQ ID NO:13 5'-attaaccctcactaaagggatatttaggtgacactatag-3'), vaRNA I-tRNA, vaRNA I-CMV, vaRNA I-RSV, vaRNA I-SV40, vaRNA I-PEPCK, vaRNA I-MT, vaRNA I-SRα, vaRNA I-P450 family, vaRNA I-GAL7, T_7-vaRNA I, T_3-vaRNA, vaRNA I-SP6, vaRNA I-K11, and vaRNA I-heat shock protein double promoters, while triple promoters are exemplified, but not limited to, the CMV-T7-vaRNA I triple promoter. The term "promoter" also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression in other tissues as well. ¶ As used herein "promoter" refers to viral, phage, prokaryotic and/or eukaryotic transcriptional control sequences. Viral promoters are exemplified by CMV, RSV, SV40, herpes simplex thymidine kinase promoter, as well as any of the various retroviral LTR promoter elements (e.g. the MMTV LTR).

Phage promoters are exemplified, but not limited to, promoters from T3 phage, SP6 phage, T7 phage, T5 phage, phage .phi.105, phage .phi.105MU331, lambda phage promoters (*e.g.*, P_{RM} and P_R). The term "promoter" includes portions of the "full length"

promoter that may be extended (*e.g.*, by PCR) to obtain a full length promoter. Thus, for example, a T7 promoter comprises 2 to 20, preferably 2 to 15, more preferably 2 to 10, yet more preferably 2 to 5 contiguous nucleotides of SEQ ID NO:1 (5'-TAATACGACTCACTATAGGG-3'). Exemplary T7 promoters may be 18 nucleotides long such as SEQ ID NO:4 (5'-TAATACGACTCACTATA-3') (see U.S. Patent Application No. 20020068290A1, to Yarovinsky, Timur, June 6, 2002), 16 nucleotides long such as SEQ ID NO:14 (5'-TAATACGACTCACTAT-3'), SEQ ID NO:15 (5'-AATACGACTCACTATA-3'), and SEQ ID NO: (5'-ATACGACTCACTATAG-3'), 14 nucleotides long such as SEQ ID NO:16 (5'-ACGACTCACTATAG-3'), SEQ ID NO:17 (5'-ATACGACTCACTAT-3'), and SEQ ID NO:19 (5'-TACGACTCACTATA-3'), and 10 nucleotides long such as SEQ ID NO:20 (5'-CGACTCACTA-3'), SEQ ID NO:21 (5'-TACGACTCAC-3'), and SEQ ID NO:22 (5'-ATACGACTCA-3'). In a preferred embodiment, the T7 promoter is a hexanucleotide exemplified by SEQ ID NO:6 (5'-ataggg-3'), (Figure 2), SEQ ID NO:23 (5'-TAATAC-3'), and SEQ ID NO:1 (5'-ACGACT-3').

In yet another example, a T3 promoter comprises 2 to 20, preferably 2 to 15, more preferably 2 to 10, yet more preferably 2 to 5 contiguous nucleotides of SEQ ID NO:8 (5'-attaaccctcactaaaggga-3'). Exemplary T3 promoters may be 18 nucleotides long such as SEQ ID NO:24 (5'-ttaaccctcactaaaggg-3'), SEQ ID NO:25 (5'-taaccctcactaaaggga-3'), and SEQ ID NO:26 (5'-attaaccctcactaaagg-3'), 16 nucleotides long such as SEQ ID NO:27 (5'-accctcactaaaggga-3'), SEQ ID NO:28 (5'-taaccctcactaaagg-3'), and SEQ ID NO:29 (5'-ttaaccctcactaaag-3'), 14 nucleotides long such as SEQ ID NO:30 (5'-cctcactaaaggga-3'), SEQ ID NO:31 (5'-attaaccctcacta-3'), and SEQ ID NO:32 (5'-aaccctcactaaag-3'), 10 nucleotides long such as SEQ ID NO:33 (5'-accctcacta-3'), SEQ ID NO:34 (5'-cctcactaaa-3'), and SEQ ID NO:35 (5'-attaaccctc-3'), and 6 nucleotides long such as SEQ ID NO:36 (5'-attaac-3'), SEQ ID NO:37 (5'-aaggga-3'), and SEQ ID NO:38 (5'-cactaa-3').

In a further example, a SP6 promoter comprises 2 to 19, preferably 2 to 15, more preferably 2 to 10, yet more preferably 2 to 5 contiguous nucleotides of SEQ ID NO:9 (5'-tatttaggtgacactatag-3'). Exemplary SP6 promoters may be 16 nucleotides long such as SEQ ID NO:39 (5'-ttaggtgacactatag-3'), SEQ ID NO:40 (5'-tttaggtgacactata-3'), and SEQ ID NO:41 (5'-tatttaggtgacacta-3'), 14 nucleotides long such as SEQ ID NO:42 (5'-atttaggtgacact-3'), SEQ ID NO:43 (5'-tatttaggtgacac-3'), and SEQ ID NO:44

(5'-tttaggtgacacta-3'), 10 nucleotides long such as SEQ ID NO:45 (5'-ttaggtgaca-3'), SEQ ID NO:46 (5'-taggtgacac-3'), and SEQ ID NO:47 (5'-atttaggtga-3'), and 6 nucleotides long such as SEQ ID NO:48 (5'-ctatag-3'), SEQ ID NO:49 (5'-tattta-3'), and SEQ ID NO:50 (5'-gacact-3').

Prokaryotic promoters include those carrying optimal -35 and -10 (Pribnow box) sequences for transcription by a prokaryotic (e.g. E. coli) RNA polymerase. In addition, some prokaryotic promoters contain overlapping binding sites for regulatory repressors (e.g. the Lac promoter and the synthetic TAC promoter, which contain overlapping binding sites for lac repressor thereby conferring inducibility by the substrate homolog IPTG). Prokaryotic genes from which suitable promoters sequences may be obtained include the E. coli lac, ara and trp genes. Further exemplary promoters include, PEPCK, MT, SRα, P450 family, GAL7, K11, and heat shock protein promoters.

The terms "functional promoter" and "promoter activity" when made in reference to a property of a nucleic acid sequence refer to the ability of the nucleic acid sequence to initiate transcription of an oligonucleotide sequence into mRNA.

The terms "cognate promoter" and "cognate promoter of RNA polymerase" refer to a promoter sequence which is a naturally occurring promoter sequence in a gene encoding the RNA polymerase. For example, the cognate promoter of T_7 RNA polymerase is the promoter which is derived from the gene encoding RNA polymerase in T_7 bacteriophage. The cognate promoter sequence may be cloned from the genome encoding the RNA polymerase. The location of a promoter may be identified by approaches and methods well known in the art, including DNase foot printing of the RNA polymerase-bound genome DNA, mutational analysis, *etc.* Alternatively, where the sequence of a cognate promoter is known, the promoter sequence may be synthesized.

The term "amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction technologies well known in the art (Dieffenbach CW and GS Dveksler (1995) *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY). As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis disclosed in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, all of which are hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of

introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

The term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis, U.S. Patent Nos. 4,683,195 and 4,683,202, hereby incorporated by reference. PCR methods are well known in the art (Dieffenbach and Dveksler (1995) *PCR Primer, a Laboratory Manual,* Cold Spring Harbor Press, Plainview, NY).

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis disclosed in U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension can be repeated many times (*i.e.*, denaturation,

annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; and/or incorporation of ^{32}P-labeled deoxyribonucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications. Amplified target sequences may be used to obtain segments of DNA (*e.g.*, genes) for the construction of targeting vectors, transgenes, etc.

The terms "flanking," and "flank" when made in reference to a first and second nucleotide sequences (*e.g.*, promoters and/or portions thereof) in relation to a third nucleotide sequence (*e.g.*, a DNA sequence of interest) mean that the first nucleotide sequence is linked to the 5' end of the third sequence, and the second nucleotide sequence is linked to the 3' end of the third sequence. For example, the configuration of left and right inverted terminal repeats of adenovirus flanking a nucleotide sequence of interest means that the left inverted terminal repeat is linked to the 5' end of the nucleotide sequence of interest, and the right inverted terminal repeat is linked to the 3' end of the nucleotide sequence of interest.

Thus, in one embodiment, the DNA sequence of interest is flanked by a promoter (and/or portion thereof) in a head to head orientation. Picturing the promoter as an arrow, the tail of the promoter refers to the 5' end that binds to RNA polymerase and the tail refers to the 3' end of the promoter to which RNA polymerase is translocated during transcription of DNA sequences that are linked to the promoter. When arranged in a "tail to tail orientation," the tails of each promoter (and/or portion thereof) face each

other and are located in proximity to one another while the heads of each promoter (and/or portion thereof) are separated and face outward. In contrast, when arranged in a "head to head orientation," the heads of each promoter (and/or portion thereof) face each other and are located in proximity to one another while the tails of each promoter (and/or portion thereof) are separated and face outward.

In another embodiment of the invention, the method further comprises: c) providing RNA polymerase that specifically binds to the first promoter; and d) contacting the first nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest. This embodiment may be useful when, for example, the promoter flanking the DNA that is produced in the first amplification steps is the functional promoter (such as the full length promoter), rather than a fragment thereof.

The term "RNA polymerase" as used herein refers to a an enzyme which catalyses the synthesis of RNA. RNA polymerase include those from a phage and from a eukaryote such as RNA polymerase I, II, and III.

As used herein, the terms "bacteriophage virus," "bacteriophage" and "phage" interchangeably refer to a bacterial virus containing a DNA core and a protective proteinaceous shell. The invention encompasses *Escherichia coli* bacteriophages such as M13 and T7. It is expressly contemplated that the invention also includes one or more bacteriophage chosen from gh-1 (ATCC 12633-B1), gh-1, Bacillus cereus phage deposited as Bacillus cereus phage (ATCC 12826-B1), HF (ATCC 15376-B1), phi V-1 (ATCC 15597-B2), PP7 (ATCC 15692-B2), PB2 (ATCC 23341-B1), Q-beta (ATCC 23631-B1), Mu-1 (ATCC 23724-B9), PM2 (ATCC 27025-B1), SPO1 (ATCC 27370-B1), P4 sid1 (ATCC 29746-B1), phage 2 designated as 2 (J1 328) (ATCC 35919-B1), phage 4 designated as 4 (J2101) (ATCC 35921-B1), phage 7 designated as 7 (L2 106) (ATCC 35922-B1), phage 12 designated as 12 (WI 3106) (ATCC 35922-B2), phage 14 designated as 14 (J2106)(ATCC 35922-B3), phage 8 designated as 8 (L2 305) (ATCC 35923-B1), phage 9 designated as 9 (WI 3263) (ATCC 35924-B1), phage 13 designated as 13 (J1 263) (ATCC 35924-B2), and phage 10 designated as 10 (L286) (ATCC 35925-B1). Also expressly contemplated are bacteriophage that is selected against one or more bacterium, such as staphylococci, hemophilii, helicobacter, mycobacterium, mycoplasmi, streptococci, neisserii, klebsiella, enterobacter, proteus, bacteriodes, pseudomonas, borrelii, citrobacter, escherichia, salmonella, propionibacterium, treponema,

shigella, enterococci, and leptospirex. Methods for selecting bacteriophage against bacteria are exemplified by those described in U.S. Patent No. 6,121,036. In one embodiment, the bacteriophage is exemplified by a) Escherichia coli bacteriophages P2, T2, T3, T4, T7, .phi.I, .phi.II, W31, H, Y, A1122, cro, C21, C22, C23, .lambda. such as lambda bacteriophage ATCC Accession no. 40143 and 40144., .phi.X174 and MS2; b) Pseudomonas putida bacteriophage gh-1; c) Salmonella typhimurium bacteriophage SP6; d) Serratia marcescens bacteriophage IV; e) Citrobacter bacteriophage ViIII; f) Klebsiella bacteriophage Number 11. Such bacteriophage are described in U.S. Patent No. 5,824,528. In another embodiment, the bacteriophage is exemplified by Salmonella bacteriophage FELIX01, Pseudomonas bacteriophage .phi.6 and PM2, and filamentous bacteriophage such as fd, M13, f1, Ifl, ZJ/2, Ff, Xf, Pfl and Pf3 (U.S. Patent No. 6,468,746). In a preferred embodiment, the phage is T7, T3, and/or SP6.

The terms "specific binding" or "specifically binding" when used in reference to the interaction of a polypeptide (such as RNA polymerase) with a nucleic acid sequence (such as a promoter) means that the interaction is dependent upon the presence of a particular structure on or within the nucleic acid sequence; in other words the polypeptide is recognizing and binding to a specific structure on or within the nucleic acid sequence rather than to nucleic acids or to nucleic acid sequences in general. For example, if a polypeptide is specific for structure "A", the presence of a nucleic acid sequence containing structure A (or free, unlabelled A) in a reaction containing labelled "A" and the polypeptide will reduce the amount of labelled A bound to the polypeptide.

Further amplification of the DNA that is flanked by one or more promoter portions may be desirable, for example where the first amplification results in a DNA sequence that is flanked by one or more non-functional portions of one or more promoter, rather than by functional portions of both promoters. Thus, in one embodiment, the method further comprises: c) providing a third primer complementary to the at least portion of the first promoter; and d) amplifying the first nucleic acid molecule produced in step b) in the presence of the third primer to produce a second nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the first promoter in a head to head orientation. This results in, for example, double stranded DNA that is now flanked by two functional promoters.

Where it is desirable to generate double stranded RNA from the resulting double stranded DNA, the method may further comprise: e) providing RNA polymerase that

specifically binds to the first promoter; f) contacting the second nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest.

Without intending to limit the source, nature, or sequence of the nucleotide sequences of interest, such sequences include, but are not limited to, coding sequences of structural genes (*e.g.*, reporter genes, selection marker genes, oncogenes, drug resistance genes, growth factors, *etc.*), and non-coding regulatory sequences which do not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*). Illustrative genomic sequences which may be used in the invention's methods include, but are not limited to, sequences which encode enzymes; lymphokines (*e.g.*, interleukins, interferons, TNF, *etc.*); growth factors (*e.g.*, erythropoietin, G-CSF, M-CSF, GM-CSF, *etc.*); neurotransmitters or their precursors or enzymes responsible for synthesizing them; trophic factors (*e.g.*, BDNF, CNTF, NGF, IGF, GMF, aFGF, bFGF, NT3, NT5, HARP/pleiotrophin, *etc.*); apolipoproteins (*e.g.*, ApoAI, ApoAIV, ApoE. *etc.*); lipoprotein lipase (LPL); the tumor-suppressing genes (*e.g.*, p53, Rb, RaplA, DCC k-rev, *etc.*); factors involved in blood coagulation (*e.g.*, Factor VII, Factor VIII, Factor IX, *etc.*); suicide genes (thymidine kinase or cytosine deaminase); blood products; hormones; *etc.*

In a further embodiment, nucleotide sequences of interest also include non-coding regulatory sequences which do not encode an mRNA or protein product, (*e.g.*, promoter sequence, polyadenylation sequence, termination sequence, enhancer sequence, *etc.*).

While not intending to limit the nucleotide sequence of interest to any particular sequence, source, or nature, such sequences are exemplified, but not limited to, adenosine deaminase (ADA) gene (GenBank Accession No. M13792); alpha-1-antitrypsin gene (GenBank Accession No. M11465); beta chain of hemoglobin gene (GenBank Accession No. NM_000518); receptor for low density lipoprotein gene (GenBank Accession No. D16494); lysosomal glucocerebrosidase gene (GenBank Accession No. K02920); hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene (GenBank Accession No. M26434, J00205, M27558, M27559, M27560, M27561, M29753, M29754, M29755, M29756, M29757); lysosomal arylsulfatase A (ARSA) gene (GenBank Accession No. NM_000487); ornithine transcarbamylase (OTC) gene (GenBank Accession No. NM_000531); phenylalanine hydroxylase (PAH) gene (GenBank Accession No.

NM_000277); purine nucleoside phosphorylase (NP) gene (GenBank Accession No. NM_000270); the dystrophin gene (GenBank Accession Nos. M18533, M17154, and M18026); the utrophin (also called the dystrophin related protein) gene (GenBank Accession No. NM_007124); and the human cystic fibrosis transmembrane conductance regulator (CFTR) gene (GenBank Accession No.M28668). Also without intending to limit the DNA sequence to any particular sequence, in one embodiment, the DNA sequence of interest comprises a sequence chosen from one or more of cDNA (Figure 2), intron (Figure 2), exon (Figure 2), expression vector Figure 1), and promoter (Figures 1 and 2)). In another embodiment, the second strand of the double stranded DNA sequence of interest comprises at least a portion of a second promoter. In a further embodiment, the second promoter is different from the first promoter. In a yet more preferred embodiment, the first promoter comprises at least a portion of a promoter chosen from T7 promoter, T3 promoter, and SP6 promoter, and the second promoter is chosen from T7 promoter, T3 promoter, and SP6 promoter.

Furthermore, nucleic acid sequences of interest include those in expression vectors.

Without intending to limit the invention to any particular promoter, in one embodiment, the first promoter comprises one or more of T7 promoter (Figure 1), T3 promoter, and SP6 promoter.

It may be desirable in some embodiments to use two hybrid promoters (such as double promoters, triple promoters, *etc.*) instead of a single promoter as illustrated by the T7 promoter in Figure 1. Thus, without intending to limit any of the primers to a particular sequence, in one embodiment, the first strand of the double stranded DNA comprises a nucleotide sequence linked to the 3' end of the first promoter, and the first primer further comprises a second sequence complementary to the nucleotide sequence, wherein the second sequence is linked to the 3' end of the first sequence of the first primer. In an alternative embodiment, the first primer comprises a sequence complementary to one or more of T7 primer (such as SEQ ID NO:1), T3 promoter (such as SEQ ID NO:8), and SP6 promoter (such as SEQ ID NO:9).

In another embodiment, the first sequence comprised on second primer is complementary to at least a portion of a promoter. Preferably, the promoter is chosen from one or more of T7 promoter, T3 promoter, and SP6 promoter. In an alternative embodiment, the second primer comprises one or more of T7-T3 primer (such as SEQ

ID NO:2 and 10), T7-SP6 (such as SEQ ID NO:3 and 11), and T3-SP6 (such as SEQ ID NO:12 and 13).

In one embodiment, the DNA sequence of interest is first cloned in a plasmid vector as known in the art. The DNA sequence of interest usually contains a full-length or partial gene sequence. Plasmid vectors used for amplification and production of the DNA sequence of interest, which contain a T7 promoter at one terminus of the cloning site and T3 or SP6 promoter at the other terminus are commercially available. In one embodiment, one strand of the dsRNA is transcribed by T7 RNA polymerase while the other strand by T3 or SP6 RNA polymerase. The two separately transcribed RNA strands are mixed in a single container and subjected to annealing.

The transcription efficiency of the T3 or SP6 system is different from that of the T7 system. Therefore, the amount of DNA transcripts produced from the T7 system is different from that of transcripts produced in the T3 or SP6 systems even from an identical DNA template. The less efficient transcription system sets the limit of overall DNA and dsRNA production. In one embodiment, the overall DNA and dsRNA preparation entails transcription reactions usually in separate containers (one for T7 and one for T3 or SP6). In addition, the annealing reaction is also performed separately. These spaciotemporally separated multiple steps hamper efficient production of the DNA sequence of interest and dsRNA.

The present invention solves many of these problems. The present invention finds use in the preparation of a large number of transcription templates from a DNA sequence already cloned in a plasmid vector, such as, but not limited to DNA sequences from a whole genome unigene collection or EST cDNA library. In an embodiment of the invention, the DNA sequence of interest is flanked by a T7 promotor on one side and by T3 or SP6 promoter on the other side, as above. *See* Figure 1.

In order to produce more of the DNA sequence of interest, two primers are used. The first primer is a T7 primer and the second primer is a hybrid primer. In one embodiment, the T7 primer comprises SEQ ID NO:1: 5'-TAATACGACTCACTATAGGG-3'. In a particular embodiment of the invention the hybrid primer is T7-T3, which comprises SEQ ID NO:2: 5'-TAATACGACTCACTATAGGGATTAACCCTCACTAAAGGGA-3'. In another embodiment of the invention, the hybrid primer is T7-SP6, which comprises SEQ ID NO: 3: 5'-TAATACGACTCACTATAGGGTATTTAGGTGACACTATAG-3'.

In an embodiment of the present invention, the polymerase chain reaction ("PCR") products that are generated by using these primer combinations contain the T7 promoter sequences at both termini in a head-to-head orientation. *See* Figure 1. This head-to-head orientation allows simultaneous transcription of both the sense and antisense strands in their respective 5' to 3' directions. One advantage of the present invention is that it eliminates the necessity of using two different RNA polymerases and enables T7 RNA polymerase to synthesize both sense and antisense strands of the dsRNA in a single container with the same efficacy for both strands.

B. Production of a DNA Sequence of Interest and Double Stranded RNA From a DNA Sequence That Is Not Linked to Additional Sequences

In the prior art, a DNA sequence of interest is typically directly amplified from a cloned plasmid, cDNA, or genomic DNA by a PCR reaction that uses two (*i.e.,* one forward and one reverse) primers which are specific for the DNA sequence of interest. In most cases, a T7 promoter sequence is linked to both of the gene-specific primers. Therefore, the forward and reverse primers have the following sequences: 5'-**TAATACGACTCACTATAGGG**NNN. . . NN-3' (T7 primer sequence in bold letters; gene-specific sequences denoted by 'N'). A consequent PCR product contains T7 promoter sequence at both termini in a head-to-head orientation. Using this PCR product as a transcription template, both sense and antisense RNA strands are transcribed by T7 RNA polymerase. Double stranded RNA is generated cotranscriptionally in the same reaction.

Unlike the present invention, the above-described prior art reaction necessitates two very long primers that contains the full sequence of the T7 promoter linked to a gene-specific sequence. In order to analyze a large number of genes (especially for genome-wide screening) a large number of primer preparations are required, which can be cost prohibitive.

To address one or more of the prior art's short falls, in one embodiment, the invention provides a method for producing a nucleic acid sequence, comprising: a) providing: i) a first double stranded DNA sequence of interest having a first strand and second strand; ii) a first primer comprising a first sequence complementary to the 3' end of the first strand, and a second sequence complementary to at least a portion of a promoter, wherein the second sequence is linked to the 5' end of the first sequence; iii) a

second primer comprising a first sequence complementary to the 3' end of the second strand, and a second sequence complementary to at least a portion of the promoter, wherein the second sequence is linked to the 5' end of the first sequence; and b) amplifying the first double stranded DNA sequence of interest in the presence of the first primer and the second primer to produce a first nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the at least a portion of the promoter in a head to head orientation.

Where the flanking promoter portions have promoter activity, it may be desirable under some embodiments to proceed with generation of RNA. Thus in one embodiment, the method further comprises: c) providing RNA polymerase that specifically binds to the promoter; and d) contacting the first nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest.

Alternatively, where one or more of the flanking promoter portions does not have promoter activity, it may be desirable to proceed with additional amplification using primers that will generate a promoter portion having promoter activity. Thus, in an alternative embodiment, the method further comprises: c) providing a third primer complementary to the at least portion of the promoter; and d) amplifying the first nucleic acid molecule produced in step b) in the presence of the third primer to produce a second nucleic acid molecule comprising the double stranded DNA sequence of interest flanked by the promoter in a head to head orientation. To generate double stranded RNA, the method further comprises: e) providing RNA polymerase that specifically binds to the promoter; f) contacting the second nucleic acid molecule with the RNA polymerase to produce double stranded RNA that is complementary to the double stranded DNA sequence of interest.

While not intending to limit the invention to any DNA sequence, in one embodiment, the DNA sequence of interest comprises a sequence chosen from one or more of cDNA (Figure 2), intron (Figure 2), exon (Figure 2), expression vector (Figure 1), and promoter (Figures 1 and 2). Also without limiting the invention to any promoter, in one embodiment, the promoter comprises one or more of T7 promoter (Figure 2), T3 promoter, and SP6 promoter. Also without intending to limit the sequence of any of the primers, in one embodiment, the second sequence of the first primer comprises SEQ ID NO:6 (Figure 2). In another embodiment, the second sequence of the second primer

comprises SEQ ID NO:6 (Figure 2). In yet a further embodiment, the third primer comprises a sequence complementary to one or more of T7 promoter (such as SEQ ID NO:1, Figure 2), T3 promoter (such as SEQ ID NO:8), and SP6 promoter (such as SEQ ID NO:9).

One embodiment of the present invention also includes a T7 promoter at both termini in a head-to-head orientation, however, the present invention does not require the cloning steps. An embodiment of the present invention includes, but is not necessarily limited to, gene-specific primers having six nucleotides ("hexanucleotide sequence") at the 5' termini instead of a full length T7 sequence. The primers of the present invention are much shorter than the ones conventionally used. *See* Figure 2. In an embodiment of the present invention, the first and second primers contain the following sequence (SEQ ID NO:6): 5'-ATAGGG-3'.

In an embodiment of the present invention a DNA sequence of interest is first amplified using PCR methods well known in the art, but using first and second primers described above (SEQ ID NO:6). *See* Example 2 *infra*. The amplified DNA sequence of interest produced is flanked by 5'-tatccc-3' (SEQ ID NO:5) at the 5' end and by 5'-ataggg-3' (SEQ ID NO:6) at the 3' end. *See* Figure 2.

In an embodiment of the present invention, the amplified DNA sequence of interest produced by the first PCR step is amplified in a second PCR step. *See* Example 2 *infra*. This second amplification is done in the presence of T7 primer (SEQ ID NO:1). While an understanding of the mechanism is not required to practice the present invention, and the present invention is not limited to any particular mechanism, the T7 primer hybridizes to the hexanucleotide sequence that was added to the DNA sequence of interest in the first PCR amplification. The DNA sequence of interest produced by this second PCR step results in a DNA sequence of interest flanked by T7 promotor sequences in a head-to-head orientation. *See* Figure 2. While an understanding of the mechanism is not required to practice the present invention, and the present invention is not limited to any particular mechanism, the shorter gene-specific primers are effective because the first PCR product is re-amplified by T7 primers in a second PCR reaction.

Unlike the present invention, conventional methods require adding the full length T7 sequence to all different gene-specific primers thereby increasing the cost of oligonucleotide synthesis due to the increased primer length. In contrast, in one embodiment, the present invention adds only six additional nucleotides (hexanucleotide

sequence). Another advantage of the present invention over the prior art is that by adding a smaller number of nucleotides to the gene-specific primers, the chances of non-specific PCR products from genomic DNA are minimized. This problem is normally seen with gene-specific primers having a long extra tail (T7 sequence).

In another embodiment of the present invention dsRNA is produced using the product of the second PCR amplification step. Preferably, in contrast to the prior art, only T7 RNA polymerase is used, rather than a combination of T7 polymerase with either T3 polymerase or SP6 polymerase, because both strands have the T7 promotor. *See* Figure 2.

C. Kits for Producing a DNA Sequence of Interest And Double Stranded RNA

The present invention also provides kits for producing a DNA sequence of interest. In some embodiments, the kits are useful in producing DNA sequences and double stranded RNA sequences. These products may be used, for example, for determining whether the subject is at risk of developing a disease or condition depending on the DNA sequence of interest by using, for example, double stranded RNA (dsRNA) mediated RNA interference (RNAi) technology (see, Example 6 herein, (Elbashir *et al.*, (2001), Ramet et al., (2002), and Hannon (2002)).

As used herein, the term "kit" is used in reference to a combination of reagents and other materials. It is contemplated that the kit may include reagents such as buffering agents, nucleic acid stabilizing reagents, protein stabilizing reagents, signal producing systems (*e.g.*, florescence generating systems as Fret systems), antibodies, control proteins, control nucleic acid sequences, as well as testing containers (*e.g.*, microtiter plates, *etc.*). It is not intended that the term "kit" be limited to a particular combination of reagents and/or other materials. In one embodiment, the kit further comprises instructions for using the reagents. The test kit may be packaged in any suitable manner, typically with the elements in a single container or various containers as necessary along with a sheet of instructions for carrying out the test. In some embodiments, the kits also preferably include a positive control sample.

Kits may be produced in a variety of ways. In some embodiments, the kits contain at least one reagent for amplifying a DNA sequence of interest. In other embodiments, the reagents are primers for amplifying DNA sequence of interest. In

some embodiments, the kit contains instructions for producing the DNA sequence of interest and/or for producing a double stranded RNA sequence encoded by the DNA sequence of interest. In preferred embodiments, the instructions specify that risk for developing a disease or condition is determined by detecting the function of an allele in the subject, wherein subjects having an allele may have an increased risk of developing a disease or condition.

In particular, the invention provides a kit comprising: a) a first primer comprising a sequence complementary to at least a portion of a first promoter (such as T7, T3, and SP6); b) a second primer comprising a first sequence complementary to at least a portion of the first promoter, and a second sequence complementary to at least a portion of a second promoter (such as T7-T3 hybrid promoter (SEQ ID NO: 2 and 10), T7-SP6 hybrid promoter (SEQ ID NO:3 and 11), and/or SP6-T3 hybrid promoter (SEQ ID NO:12 and 13)), wherein the first and second promoters are different; and c) instructions for producing a double stranded DNA sequence flanked by the first promoter in a head to head orientation or flanked by the second promoter in a head to head orientation, using the first primer and the second primer. In one embodiment, the kit further comprises: d) providing RNA polymerase that specifically binds to one or more of the first promoter and the second promoter; and e) instructions for producing a double stranded RNA sequence complementary to the DNA sequence using the RNA polymerase. While not limiting the invention to any particular promoter, in one embodiment, the first promoter comprises a sequence chosen from one or more of T7 promoter (SEQ ID NO:1):, T3 promoter (SEQ ID NO:8), and SP6 promoter (SEQ ID NO:9). In another embodiment, the second promoter comprises a sequence chosen from one or more of T7 promoter (SEQ ID NO:1):, T3 promoter (SEQ ID NO:8), and SP6 promoter (SEQ ID NO:9). Also without limiting the primers to any particular sequence, in one embodiment, the second primer comprises a sequence chosen from one or more of T7-T3 primer (such as SEQ ID NO: 2 and 10), T7-SP6 primer (such as SEQ ID NO:3 and 11), and SP6-T3 hybrid primer (such as SEQ ID NO:12 and 13).

Also provided herein is a kit for producing a nucleic acid sequence, comprising: a) a first primer comprising a sequence complementary to at least a portion of a promoter; b) a second primer comprising a sequence complementary to at least a portion of the promoter; and c) instructions for producing a double stranded DNA sequence

flanked by the first promoter in a head to head orientation using the first primer and the second primer.

In one embodiment, it may be desirable to use first and second primers that are the same, for example, where the primers are complementary to the same portion of the same promoter. Alternatively, it may be desirable to use first and second primers that are different, for example, where the primers are complementary to different portions of the same promoter.

In an alternative embodiment, the kit further comprises: d) providing RNA polymerase that specifically binds to the promoter; and e) instructions for producing a double stranded RNA sequence complementary to the DNA sequence using the RNA polymerase. Alternatively, the kit further comprises: d) providing a third primer comprising a sequence complementary to the promoter, wherein the third primer is different from the first and second primers; and e) instructions for producing a double stranded DNA sequence flanked by the third promoter in a head to head orientation using the third primer. More preferably, the kit further comprises: f) providing RNA polymerase that specifically binds to the promoter; and g) instructions for producing a double stranded RNA sequence complementary to the DNA sequence using the RNA polymerase.

While not limiting the promoter to any particular sequence, the promoter comprises a sequence chosen from one or more of T7 promoter (SEQ ID NO:1):, T3 promoter (SEQ ID NO:8), and SP6 promoter (SEQ ID NO:9).

In a further embodiment, the invention provides a kit for producing a DNA sequence of interest flanked by T7 polymerase promoter in a head to head orientation, comprising: a) a first primer complementary to a T7 promoter, the first primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); b) a second primer complementary to a T7-T3 hybrid promoter, the second primer comprising 5'-TAATACGACTCACTATAGGGATTAACCCTCACTAAAGGGA-3' (SEQ ID NO: 2); c) instructions for producing the DNA sequence of interest using the first primer and the second primer.

In an alternative embodiment, the invention provides a kit for producing a DNA sequence of interest flanked by T7 polymerase promoter in a head to head orientation, comprising: a) a first primer complementary to a T7 promoter, the first primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); b) a second primer

complementary to a T7-SP6 hybrid promoter, the second primer comprising 5'-TAATACGACTCACTATAGGGTATTTAGGTGACACTATAG-3' (SEQ ID NO:3); and c) instructions for producing the DNA sequence of interest using the first primer and the second primer.

In an additional embodiment, the invention provides a kit for producing a DNA sequence of interest flanked by T7 polymerase promoter in a head to head orientation, comprising: a) a first primer comprising hexanucleotide 5'-ataggg-3' (SEQ ID NO:6) at the 3' end of the first primer; b) a second primer comprising hexanucleotide 5'-ataggg-3' (SEQ ID NO:6) at the 5' end of the second primer; c) a third primer complementary to a T7 promoter, the third primer comprising 5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO:1); and d) instructions for producing the DNA sequence of interest using the first primer, the second primer, and the third primer.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μM (micromolar); N (Normal); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μg (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers); nm (nanometers); °C (degrees Centigrade); U (units), mU (milliunits); min. (minutes); sec. (seconds); and % (percent).

EXAMPLE 1

Preparation of a Transcription Template from a DNA Sequence of Interest Cloned in a Plasmid Vector

A cDNA or exon fragment with a target gene sequence was cloned in a conventional plasmid vector at the multicloning site flanked by T7 and SP6 (*e.g.*, pCR brand II-TOPO plasmid vector; Invitrogen Corp., Carlsbad, CA) or T7 and T3 (*e.g.*, pBluescript brand II phagemid vector; Stratagene, La Jolla, CA). Transcription templates for *in vitro* transcription were prepared from this plasmid by polymerase chain reaction ("PCR") using the T7 (SEQ ID NO: 1) and T7-SP6 (SEQ ID NO:2) primer pair or the

T7 and T7-T3 (SEQ ID NO:3) primer pair. The resulting PCR products have a T7 sequence at both termini in a head-to-head orientation. (*See* Figure 1).

The PCR reaction mixture (final 20 µl total volume) contained 10 ng of plasmid template, 1X Taq buffer, 5 mM $MgCl_2$ (magnesium chloride), 1mM deoxyribonucleoside triphosphate ("dNTP"), 20 pM T7 primer, 20 pM of T7-SP6 primer or T7-T3 primer, Taq polymerase (standard unit as recommended by manufacturer). The reaction mixture was heated at 94°C for 5 minutes, then 40 cycles of (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 60-90 seconds), and for 72°C for 5 minutes.

EXAMPLE 2

Preparation of Transcription Template from a DNA Sequence of Interest Without A Cloning Procedure

A DNA sequence with a target gene sequence was directly amplified from either a cDNA pool or genomic DNA using a pair of gene-specific forward and reverse primers. The gene-specific primers had the sequence that is at least partially complementary to the target gene sequence as well as hexanucleotide 5'-tail sequence (ataggg) that is identical to the last six nucleotide sequence of T7 primer. After amplification with these forward and reverse primers, the resulting PCR products was expected by the inventors to have the hexanucleotide sequence at both termini in a head-to-head orientation. The position of the gene-specific primer sequence may be chosen in such a way that a part of the hexanucleotide sequence on the 3' side overlaps with a part of the gene-specific sequence on the 5' side in order to reduce the length of the entire primer.

In the first PCR reaction mixture, (final 20 µl total volume) 10 ng of plasmid template, 1X Taq buffer, 5 mM $MgCl_2$, 1mM dNTP, 40 pM gene specific primer, and Taq polymerase (standard unit as recommended by manufacturer) was mixed. The reaction mixture was heated at 94°C for 5 minutes, then 40 cycles of (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 60-90 seconds), and for 72°C for 5 minutes.

After completing the first PCR amplification, the second PCR amplification was done using the first reaction's amplified DNA as a template. The second amplification reaction contained (final 20 µl total volume) 0.01 µl of the first PCR reaction mixture, 1 X Taq buffer, 5 mM $MgCl_2$, 1mM dNTP, 20 pM T7 primer, and Taq polymerase (standard unit as recommended by manufacturer). The reaction mixture was heated at

94°C for 5 minutes, then 50 cycles of (94°C for 30 seconds, 15°C for 30 seconds, 72°C for 60-90 seconds), then 35 cycles of (94°C for 30 seconds, 50°C for 30 seconds, 72°C for 60-90 seconds), and for 72°C for 5 minutes.

During the second PCR reaction, the T7 primer hybridized to the terminal hexanucleotide sequence of the first PCR product and generated a product that contained a T7 promoter sequence at both termini in a head-to-head orientation. These T7 promoter sequences then allowed transcription with T7 RNA polymerase in both directions to form the dsRNA.

EXAMPLE 3

In Vitro Transcription by T7 RNA Polymerase (ds RNA Synthesis)

Both sense and antisense RNA strands were transcribed in a single T7 transcription reaction. Most of the strands were annealed cotranscriptionally. In order to increase the annealing efficiency, the MEGAscript T7 Kit (Ambion, Inc., Austin, TX) was used according to the manufacturer's instructions in a total volume of 100 µl using 20 µl of unpurified PCR reaction (see Example 2, second amplification). The reaction was incubated for 6 hours at 37°C. Then an equal volume (50 µl) of 2x annealing buffer (40mM HEPES-KOH buffer at pH 7.6, 10mM EDTA) was added to the transcription reaction. The reaction was heated to 65°C for 30 minutes. The reaction was then slowly cooled to room temperature over a period of 2 hours. Then, 100 µl of 5M ammonium acetate and 500 µl was added to the reaction and thoroughly mixed and incubated at room temperature for 5 minutes. The reaction vessel was then centrifuged for 15 minutes at 15000 rpm at room temperature to recover the dsRNA. The supernatant was then decanted, a 70% ethanol wash was added to the pellet, centrifuged as above again and allowed to air dry. The pellet was then dissolved in 100 µl H_2O. A microliter of the final solution was analyzed by electrophoresis in a 1% agarose gel using methods well known in the art for analyzing dsDNA.

EXAMPLE 4

Transfection of dsRNA into S2 Cells

Drosophila S2 cells were cultured at 22°C in Schneider's Drosophila medium (GIBCO) supplemented with 10% fetal bovine serum FBS and antibiotics. Transfection

was carried out in either 6-well or 24-well plate format (using 2ml or 0.4 ml culture volumes, respectively).

For a transfection performed in a 24-well plate, 2 μg dsRNA mixed in 2 μl water with 5 μl LIPOFECTING for each well the cocktail is to be added was prepared and incubated for 20 minutes at room temperature. Meanwhile, S2 cells were washed with 0.4 ml Schneider's Drosophila medium with no supplementation. Double stranded RNA-lipofectin complex was added to each well and mixed gently (7 μl per well). The plate was then incubated for 6 hours at 22°C. The medium as then replaced with fully supplemented medium and incubated at 22°C.

Alternatively, the transfection could also be performed without using lipofectin or other lipid transfection reagent. For a 24 well plate, the S2 cells were washed with 0.4 ml unsupplemented medium. Then 0.1 ml of unsupplemented medium was added to each well. 5-20 μg dsRNA was diluted in water as above for each well, but without LIPOFECTIN. The dsRNA was added to each well, mixed gently, and incubated for 6 hours at 22°C. After incubating, the medium was replaced with 0.4 ml of supplemented medium and incubated at 22°C. In most cases, RNAi effect is maximized around day 2 through 4 after transfection with dsRNA.

EXAMPLE 5

Gene Silencing in *Drosophila*

RNAi was induced in *Drosophila* S2 cells and its effect was analyzed by checking the level of target proteins by Western blotting. Target proteins were almost completely removed and hardly detected after treatment with the cognate dsRNA, but not other dsRNAs. IKK, p38, and GSK3 are protein kinases well known in the mouse, but RNAi was performed with their *Drosophila* homologs. *Drosophila* S2 cells were cultured in a 24-well plate (0.4 ml culture volume per well) and were transfected with 2 μg of dsRNAs per well that were prepared by the dsRNA synthesis method as described *supra* utilizing hybrid primers. After 72 hours, total protein from transfected cells was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis ("SDS-PAGE") and analyzed by Western immunoblot analysis with DmIKK1γ, Dmp38b, DmGSK3β antibodies. *See* Figure 3, left hand side of gel. Double stranded RNAs used in this experiment were indicated on the top and made from the full length cDNAs of *Drosophila* homologs of IKKγ(DmIKK1γ/Kenny), p38 (Dmp38a and Dmp38b), and

GSK3β (DmGSK3β/Shaggy). Mock dsRNA as a control was made from a mouse IκMα cDNA sequence. Each corresponding dsRNA resulted in ablation of cognate target proteins without affecting the other proteins. In addition, cotreatment of two different dsRNAs resulted in ablation of their targets simultaneously (*see* Figure 3, lane 5).

EXAMPLE 6

Effect of Mock dsRNA versus dsRNA on S2 Cells

The elimination of target genes was analyzed by measuring the level of target (*Drosophila* Toll-1) mRNA after specific RNAi had been induced. The mRNA levels were quantitatively determined by realtime PCR analysis. *Drosophila* S2 cells were transfected with either control Mock dsRNA or *Drosophila* Toll-1 dsRNA synthesized by the method described *supra* using primers comprising the hexanucleotide sequence. After 72 hours, total RNA from transfected cells were isolated and the mRNA level of Toll-1 was analyzed by realtime PCR analysis. Treatment of S2 cells with Toll-1 dsRNA resulted in a decrease of its mRNA in cells 93% off the original level. *See* Figure 4.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, genetics, or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method for producing a nucleic acid sequence, comprising:

a) providing:

i) a first double stranded DNA sequence of interest having a first strand and second strand, and comprising a first promoter;

ii) a first primer comprisinga first sequence complementary to the 3' end of at least a portion of said first promoter that is comprised on said first strand;

iii) a second primer comprising a first sequence complementary to the 3' end of said second strand, and a second sequence complementary to at least a portion of said first promoter, wherein said second sequence is linked to the 5' end of said first sequence; and

b) amplifying said first double stranded DNA sequence of interest in the presence of said first primer and said second primer to produce a first nucleic acid molecule comprising said double stranded DNA sequence of interest flanked by said at least a portion of said first promoter in a head to head orientation.

2. The method of Claim 1, further comprising:

c) providing RNA polymerase that specifically binds to said first promoter; and

d) contacting said first nucleic acid molecule with said RNA polymerase to produce double stranded RNA that is complementary to said double stranded DNA sequence of interest.

3. The method of Claim 1, further comprising:

c) providing a third primer complementary to said at least portion of said first promoter; and

d) amplifying said first nucleic acid molecule produced in step b) in the presence of said third primer to produce a second nucleic acid molecule comprising said double stranded DNA sequence of interest flanked by said first promoter in a head to head orientation.

4. The method of Claim 3, further comprising:

e) providing RNA polymerase that specifically binds to said first promoter;

f) contacting said second nucleic acid molecule with said RNA polymerase to produce double stranded RNA that is complementary to said double stranded DNA sequence of interest.

5. The method of Claim 1, wherein said second strand of said double stranded DNA sequence of interest comprises at least a portion of a second promoter.

6. The method of Claim 5, wherein said second promoter is different from said first promoter.

7. The method of Claim 1, wherein said first promoter comprises T7 promoter.

8. The method of Claim 1, wherein said first promoter comprises T3 promoter.

9. The method of Claim 1, wherein said first promoter comprises SP6 promoter.

10. The method of Claim 1, wherein said first strand of said double stranded DNA comprises a nucleotide sequence linked to the 3' end of said first prmoter, and said first primer further comprises a second sequence complementary to said nucleotide sequence, wherein said second sequence is linked to the 3' end of said first sequence of said first primer.

11. The method of Claim 1, wherein said first primer comprises a sequence complementary to T7 promoter.

12. The method of Claim 1, wherein said first primer comprises a sequence complementary to T3 promoter.

13. The method of Claim 1, wherein said first primer comprises a sequence complementary to SP6 promoter.

14. The method of Claim 1, wherein said first sequence comprised on second primer is complementary to at least a portion of a promoter.

15. A method for producing a nucleic acid sequence, comprising:

a) providing:

i) a first double stranded DNA sequence of interest having a first strand and second strand;

ii) a first primer comprising a first sequence complementary to the 3' end of said first strand, and a second sequence complementary to at least a portion of a promoter, wherein said second sequence is linked to the 5' end of said first sequence;

iii) a second primer comprising a first sequence complementary to the 3' end of said second strand, and a second sequence complementary to at least a portion of said promoter, wherein said second sequence is linked to the 5' end of said first sequence; and

b) amplifying said first double stranded DNA sequence of interest in the presence of said first primer and said second primer to produce a first nucleic acid molecule comprising said double stranded DNA sequence of interest flanked by said at least a portion of said promoter in a head to head orientation.

16. The method of Claim 15, further comprising:

c) providing RNA polymerase that specifically binds to said promoter; and

d) contacting said first nucleic acid molecule with said RNA polymerase to produce double stranded RNA that is complementary to said double stranded DNA sequence of interest.

17. The method of Claim 15, further comprising:

c) providing a third primer complementary to said at least portion of said promoter; and

d) amplifying said first nucleic acid molecule produced in step b) in the presence of said third primer to produce a second nucleic acid molecule comprising said double stranded DNA sequence of interest flanked by said promoter in a head to head orientation.

18. The method of Claim 17, further comprising:

e) providing RNA polymerase that specifically binds to said promoter;

f) contacting said second nucleic acid molecule with said RNA polymerase to produce double stranded RNA that is complementary to said double stranded DNA sequence of interest.

19. A kit comprising:

a) a first primer comprising a sequence complementary to at least a portion of a first promoter;

b) a second primer comprising a first sequence complementary to at least a portion of said first promoter, and a second sequence complementary to at least a portion of a second promoter, wherein said first and second promoters are different; and

c) instructions for producing a double stranded DNA sequence flanked by said first promoter in a head to head orientation or flanked by said second promoter in a head to head orientation, using said first primer and said second primer.

20. The kit of Claim 19, further comprising:

d) providing RNA polymerase that specifically binds to one or more of said first promoter and said second promoter; and

e) instructions for producing a double stranded RNA sequence complementary to said DNA sequence using said RNA polymerase.

21. A kit for producing a nucleic acid sequence, comprising:

a) a first primer comprising a sequence complementary to at least a portion of a promoter;

b) a second primer comprising a sequence complementary to at least a portion of said promoter; and

c) instructions for producing a double stranded DNA sequence flanked by said first promoter in a head to head orientation using said first primer and said second primer.

22. The kit of Claim 21, wherein said first and second primers are the same.

23. The kit of Claim 21, wherein said first and second primers are different.

24. The kit of Claim 21, further comprising:

d) providing RNA polymerase that specifically binds to said promoter; and

e) instructions for producing a double stranded RNA sequence complementary to said DNA sequence using said RNA polymerase.

25. The kit of Claim 21, further comprising:

d) providing a third primer comprising a sequence complementary to said promoter, wherein said third primer is differnt from said first and second primers; and

e) instructions for producing a double stranded DNA sequence flanked by said third promoter in a head to head orientation using said third primer.

26. The kit of Claim 25, further comprising:

f) providing RNA polymerase that specifically binds to said promoter; and

g) instructions for producing a double stranded RNA sequence complementary to said DNA sequence using said RNA polymerase.

FIGURE 1

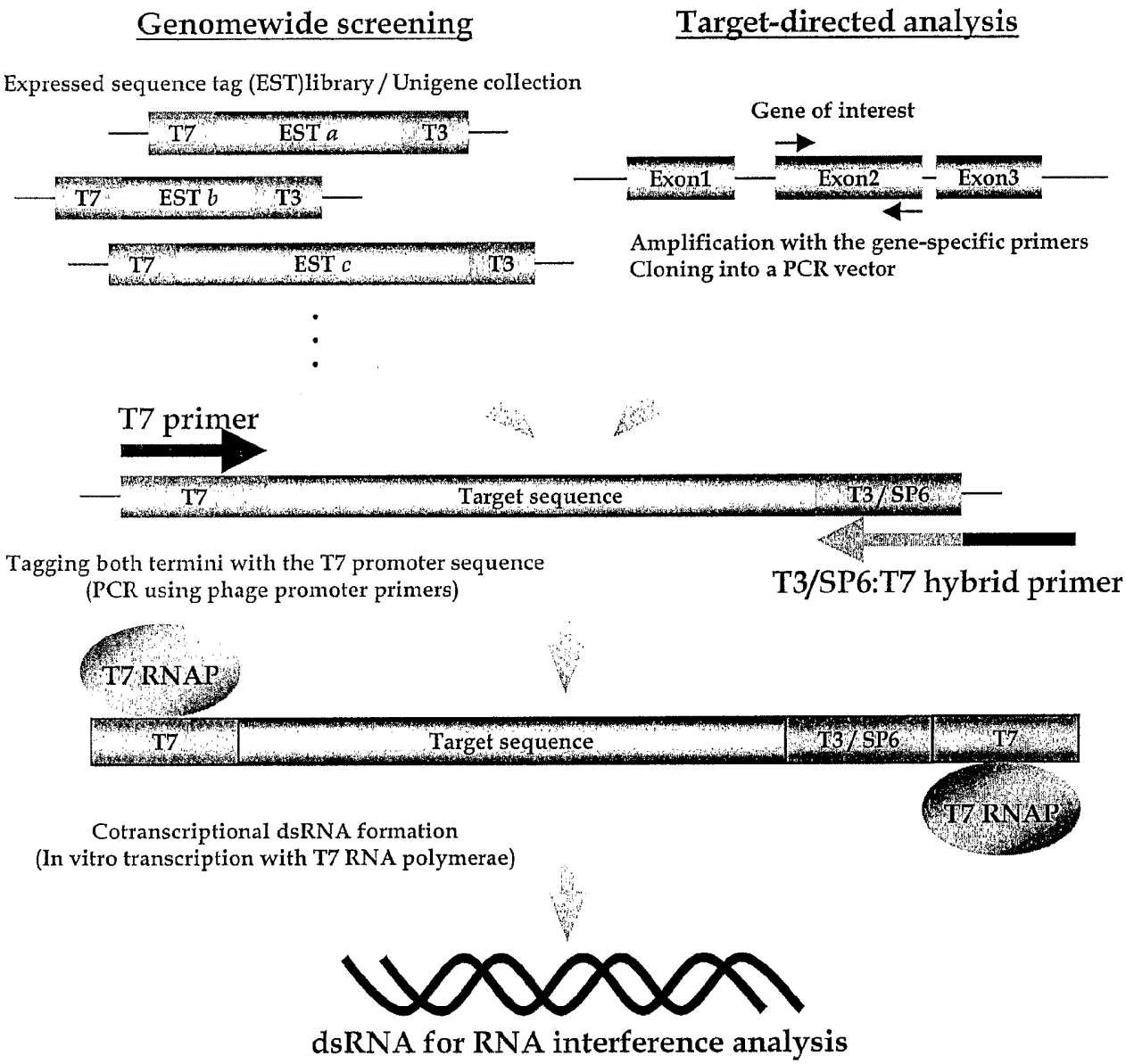
Genomewide screening
Target-directed analysis
Expressed sequence tag (EST)library / Unigene collection
T7
EST a
T3
T7
EST b
T3
T7
EST c
T3
Gene of interest
Exon1
Exon2
Exon3
Amplification with the gene-specific primers
Cloning into a PCR vector
T7 primer
T7
Target sequence
T3/SP6
Tagging both termini with the T7 promoter sequence
(PCR using phage promoter primers)
T3/SP6:T7 hybrid primer
T7 RNAP
T7
Target sequence
T3/SP6
T7
T7 RNAP
Cotranscriptional dsRNA formation
(In vitro transcription with T7 RNA polymerae)
dsRNA for RNA interference analysis

FIGURE 2

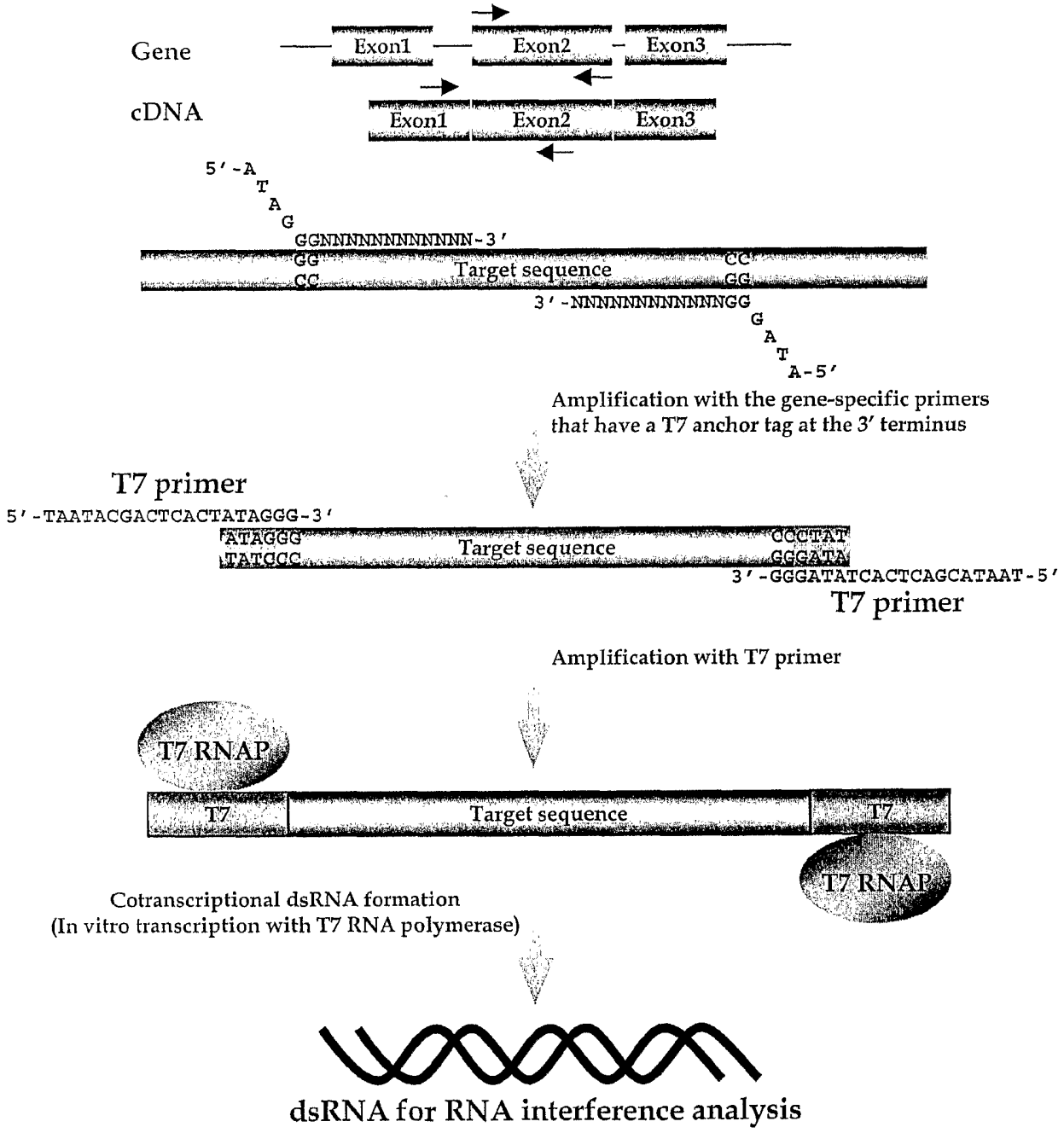
Gene
Exon1
Exon2
Exon3
cDNA
Exon1
Exon2
Exon3
5′-A
T
A
G
GGNNNNNNNNNNNN-3′
GG
CC
Target sequence
CC
GG
3′-NNNNNNNNNNNNGG
G
A
T
A-5′
Amplification with the gene-specific primers
that have a T7 anchor tag at the 3′ terminus
T7 primer
5′-TAATACGACTCACTATAGGG-3′
ATAGGG
TATCCC
Target sequence
CCCTAT
GGGATA
3′-GGGATATCACTCAGCATAAT-5′
T7 primer
Amplification with T7 primer
T7 RNAP
T7
Target sequence
T7
T7 RNAP
Cotranscriptional dsRNA formation
(In vitro transcription with T7 RNA polymerase)
dsRNA for RNA interference analysis

Figure 3

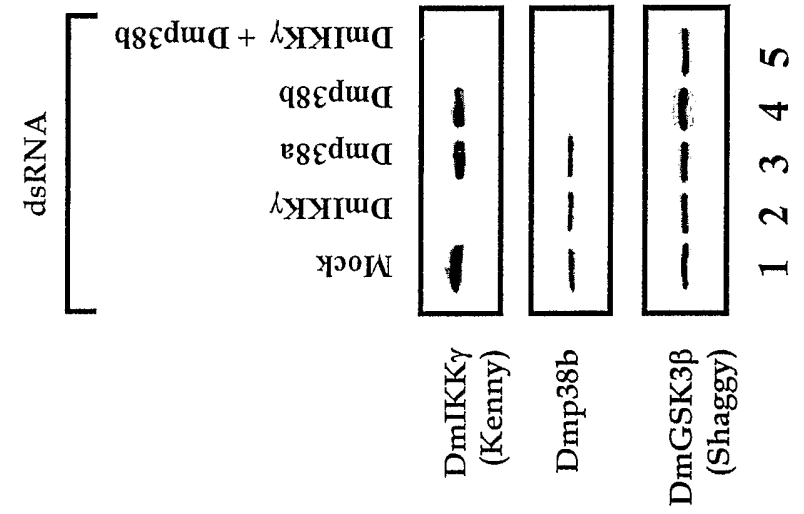
dsRNA
Mock
DmIKKγ
Dmp38a
Dmp38b
DmIKKγ + Dmp38b
DmIKKγ (Kenny)
Dmp38b
DmGSK3β (Shaggy)
1 2 3 4 5

FIGURE 4