PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, C12P 19/34 — A1

(11) International Publication Number: WO 95/00667

(43) International Publication Date: 5 January 1995 (05.01.95)

(21) International Application Number: PCT/US94/06855

(22) International Filing Date: 16 June 1994 (16.06.94)

(30) Priority Data:

08/078,759	17 June 1993 (17.06.93)	US
08/153,536	17 November 1993 (17.11.93)	US

(71) Applicant: THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK [US/US]; Suny Plaza, Broadway, Albany, NY 12210 (US).

(72) Inventors: LANE, Michael, J.; 3044 Claybourne Lane, Baldwinsville, NY 13027 (US). BENIGHT, Albert, S.; 1630 Valley View Drive, Schaumburg, IL 60193 (US). FALDASZ, Brian, D.; 4 Bellevue Terrace, Maynard, MA 01754 (US).

(74) Agents: MYERS, Paul, Louis et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: IMPROVED METHODS FOR DETECTING NUCLEIC ACID SEQUENCES

(57) Abstract

Improved cycling probe reactions are disclosed.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

IMPROVED METHODS FOR DETECTING NUCLEIC ACID SEQUENCES

Background of the Invention

The invention relates to the use of cycling probe reactions to detect the presence of nucleic acid sequences.

Numerous methods are known to the art for detecting the presence of specific nucleic acid sequences in a sample. One such method is the Cycling Probe Reaction (CPR). In this reaction, a probe specific for a sequence to be identified hybridizes to a target single strand nucleic acid to form a duplex nucleic acid. The probe includes a scissile link which becomes susceptible to cleavage upon formation of the duplex. For example, a DNA probe can include an RNA sequence segment. Formation of a duplex between such a probe and a single strand DNA molecule results in an RNA:DNA hybrid duplex. RNase H, which cleaves the RNA strand of an RNA:DNA hybrid duplex, is used to cleave the target-bound probe. Detection is based on this hybridization-specific cleavage. The cleaved probe disassociates from the target and the target can enter the cycle with a new, uncleaved, probe.

CPR technologies are described in Duck et al. U.S. Patent No. 4, 876,187, issued October 24, 1989, hereby incorporated by reference, and in Duck et al. U.S. Patent No. 5,011,069, issued April 30, 1991, hereby incorporated by reference.

Summary of the Invention

The invention features a method for detecting a single-stranded target nucleic acid. The method includes:

a. providing a reaction mixture which includes the target nucleic acid, a complementary single-stranded nucleic acid probe, the probe being present in molar excess relative to the target and having the structure $[NA_1\text{-}R\text{-}NA_2]_n$ wherein NA_1 and NA_2 are DNA sequences, wherein R is a scissile nucleic acid linkage, and wherein n is an integer from 1 to 10, and RNase H, the RNase H being present at a chemical potential sufficient to substantially increase the rate of duplex formation over what would be formed in the absence of the RNase H, and allowing target-probe duplex to form;

b. treating the target-probe duplex from step (a) so as to cleave the probe within a predetermined sequence of the scissile nucleic acid linkage and thereby form at least one intact DNA-containing oligonucleotide fragment from the probe, such fragment being, or being treated so as to be, no longer capable of remaining hybridized to the target nucleic acid;

c. repeating the cycle of steps (a) and (b); and

d. detecting the intact DNA-containing fragments so formed and thereby detecting the single-stranded target nucleic acid.

Also included in the invention is a reaction mixture which includes:

a single-stranded target nucleic acid;

a complementary single-stranded nucleic acid probe, the probe being present in molar excess relative to the target and having the structure $[NA_1\text{-}R\text{-}NA_2]_n$ wherein NA_1 and NA_2 are DNA sequences, wherein R is a scissile nucleic acid linkage, and wherein n is an integer from 1 to 10; and

a RNase H, the RNase H being present at a chemical potential sufficient to substantially increase the rate of duplex formation over what would be formed in the absence of the RNase H.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Detailed Description

The drawings are first briefly described.

Drawings

Fig. 1 is a diagram of an improved CPR.

Fig. 2 is a depiction of a gel showing the products of an improved CPR.

Tm, as used herein, refers to the midpoint of the duplex to single strand melting transition.

The chemical potential of a reagent, as used herein, refers to the free energy change of a reaction mix when the reagent is added to the reaction mixture. Chemical potential is a more exact measure of the activity of a species in a given reaction under a given set of conditions and takes into account considerations such as the number a sites a species can react with and whether all molecules of a species are available for reactions. The chemical potential of the species can usually be most directly manipulated by changing the concentrations of a species. The unit of chemical potential is free-energy, e.g., of cal/mole or J/mol, but in the methods described herein determination of an absolute chemical potential is not required. Most of the methods disclosed herein require reaction mixtures in which the ratio of chemical potential between two species, or the difference between the chemical potential of two species, is required to be such that the chemical potential of the RNase H is sufficient to drive the reaction towards the duplex.

Purified nucleic acid, as used herein, refers to a purified DNA or RNA. Purified DNA, as used herein, refers to DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (i.e., one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, e.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Purified RNA, as used herein, refers to an RNA which is substantially free of another RNA sequence with which it is found in a cell which produces the RNA. A non-

naturally occurring nucleic acid sequence is purified when it is substantially free of other sequences.

Purified natural product, as used herein, refers to a product which is produced by an organism and which is substantially free of a macromolecule, e.g., a protein or a nucleic acid, with which it occurs in an organism from which it is derived.

Product which does not naturally occur in living cells, as used herein, refers to a product which is not synthesized or produced by living cells or organisms.

Methods of the invention allow CPR reactions to be driven by RNase H. As such, the rate of the reaction is controlled by the concentration or chemical potential of the RNase and not the probe or target nucleic acids.

CPR Probes

The making and using of CPR probes is known to the art, see e.g., Duck et al. U.S. Patent No. 4, 876,187 or Duck et al. U.S. Patent No. 5,011,069.

The nucleic acid probe which is useful in the practice of this invention comprises the structure:

$$[NA_1\text{-}R\text{-}NA_2]_n$$

wherein NA_1 and NA_2 are nucleic acid sequences, wherein R is an RNA sequence; and wherein n is an integer from about 1 to about 10.

In one embodiment of this invention, NA_1 and NA_2, in the nucleic acid probe independently comprise from about 0 to about 20 nucleotides, R comprises from about 1 to about 100 ribonucleotides, or more, and n is an integer from about 1 to about 10.

In another embodiment of this invention, NA_1 and NA_2 in the nucleic acid probe are DNA sequences. In a further embodiment , NA_1 and NA_2 in the nucleic acid probe are RNA sequences. In still yet another embodiment, the nucleic acid probe comprises a structure wherein NA_1 is either an RNA or DNA sequence.

In one embodiment, nicking the hybridized probe at predetermined RNA sequences is carried out with a double-stranded ribonuclease. Such ribonucleases nick or excise ribonucleic acid sequences from double-stranded DNA-RNA hybridized stands. An example of a ribonuclease useful in the practice of this invention is RNase H. Other ribonucleases and enzymes may be suitable to nick or excise RNA from RNA-DNA strands, such as Exo III and reverse transcriptase.

The molecules of the present invention may have a detectable marker attached to one or more of the nucleic acid sequences, NA_1 or NA_2. This marker is contemplated to be any molecule or reagent which is capable of being detected. Examples of such detectable molecules are radioisotopes, radiolabelled molecules, fluorescent molecules, fluorescent antibodies, enzymes, or chemiluminescent catalysts. Another suitable marker is a ligand capable of binding to specific proteins which have been tagged with an enzyme, fluorescent

molecule or other detectable molecule. One example of a suitable ligand is biotin, which will bind to avidin or streptavidin.

In one embodiment of this invention, the nucleic acid probe is immobilized on a solid support. In another, the nucleic acid probe is labeled with a detectable marker.

When the nucleic acid sequences, NA_1 and NA_2 are DNA, the R portion described above may also be properly termed a scissile linkage in language consistent with usage in U.S. Patent No. 4,876,187. Such a linkage is capable of being cleaved or disrupted without cleaving or disrupting any nucleic acid sequence of the molecule itself or of the target nucleic acid molecule. As used herein, such a scissile linkage, i.e., R, is any connecting chemical structure which joins two nucleic acid sequences and which is capable of being selectively cleaved without cleavage of the nucleic acid sequences to which it is joined. The scissile linkage may be single bond or a multiple unit sequence. As used herein, R denotes a ribonucleic acid (RNA) sequence.

Improved Cycling Probe Reactions

Fig. 1 describes improvements on a reaction scheme often referred to as a Cycling Probe Reaction (CPR). In this reaction a probe hybridizes to a target to form a duplex. The probe includes a scissile link, which becomes susceptible to cleavage upon formation of a duplex. For example, the probe can have an RNase sensitive segment. Formation of a duplex between such a probe and a single strand DNA molecule results in an RNA:DNA hybrid duplex. An enzyme which cleaves RNA:DNA hybrids, e.g., RNase H, cleaves the probe only when it is bound to the target. Detection is based on this hybridization-specific cleavage. RNase binds the duplex to form a duplex: RNase complex. Cleavage results in a cleaved probe:target complex. The cleaved probe disassociates from the target and the target can enter the cycle with a new, uncleaved, probe.

Methods of the invention can be used to improve the performance of this reaction. As often used, this scheme is inappropriate for detecting a target sequence when insufficient RNase H is present. Traditionally, probe concentration has been increased in attempts to drive the basic reaction to the right. As described herein, increasing the chemical potential of RNase H in the reaction mix will also favor the formation of duplex. Since RNaseH dictates duplex:RNaseH complex formation increasing the potential of this species in the reaction mix will favor generation of duplex substrate and therefore of cleaved-probe (signal). Thus, the addition of RNase H can be used to increase sensitivity.

The method can be used for detecting a single-stranded target nucleic acid. In one embodiment, a reaction mixture is provided which includes the target nucleic acid, a complementary single-stranded nucleic acid probe, the probe being present in molar excess relative to the target and having the structure

$$[NA_1\text{-}R\text{-}NA_2]_n$$

wherein NA_1 and NA_2 are DNA sequences, wherein R is a scissile nucleic acid linkage, and wherein n is an integer from 1 to 10, and RNase H. The RNase H is present at a chemical potential sufficient to substantially increase the rate of duplex formation over what would be formed in the absence of the RNase H. The mixture is maintained under conditions which allow target-probe duplex to form. The target-probe duplex from step (a) is treated so as to cleave the probe within a predetermined sequence of the scissile nucleic acid linkage and thereby form at least one intact DNA-containing oligonucleotide fragment from the probe, such fragment being, or being treated so as to be, no longer capable of remaining hybridized to the target nucleic acid. The cycle of steps (a) and (b) are then repeated and the intact DNA containing fragments so formed are detected to thereby detect the single-stranded target nucleic acid.

In one certain embodiment, the method is performed at a temperature above the Tm of the duplex. For example, the temperature can be at least x C°, wherein x is 5, 10, 20, 30, 40, 50, 60, 70, or 80, above said Tm.

In preferred embodiments the rate of duplex formation is increased by at least y fold, wherein y is 2, 5, 10, 50, 100, 500, 10^3, 10^4, 10^5, 10^6, by the addition of RNase H. The RNase H can be used to drive formation of duplex under conditions where the rate of duplex formation in the absence of RNase H would be substantially zero.

In various embodiments of this invention, the concentration, number of molecules of, or the chemical potential of, the RNase H is greater than the concentration, number of molecules present, or chemical potential of the probe, the target, both the probe and the target, the duplex, or the combination of the probe, the target, and the duplex, e.g., at least z fold greater, wherein z is at least 1, 2, 5, 10, 25, 50, 100, 500, 10^3, 10^4, 10^5 or, 10^6.

In certain embodiments, the concentration, number of molecules of, or the chemical potential of, the RNase H is sufficient to allow: detection of one target molecule in a biological sample, preferably with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction, detection of a target strand at a concentration of 10^{-5} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-6} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-7} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-8} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-9} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-10} pMole or less, preferably the detection being carried out with a CPR probe labeled with an

activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-11} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-12} pMole or less, preferably the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/μl, using 20,000 cpm per reaction.

The probe and the target can be present in substantially equal amount, concentration, number, or chemical potentials in said reaction mix. The ratio by weight, molarity, number, concentration, or chemical potential of the probe to the target can be less than or equal to 1:1, 2:1, 5:1, 10:1, 25:1, 50:1, 100:1, or 10^m:1, wherein m is an integer between 3 and 10, inclusive. The ratio by weight, molarity, number, concentration, or chemical potential of the RNase H to the single strand in the highest concentration can be greater than 1:1, 2:1, 5:1, 10:1, 25:1, 50:1, 100:1, or 10^m:1, wherein m is an integer between 3 and 10, inclusive.

In preferred embodiments the RNase H will accelerate the rate of duplex formation at least n-fold, wherein n is 2, 5, 10, 50, 100, 500, 10^3, 10^4, 10^5, 10^6.

Methods and compositions of the invention are illustrated in the following example.

Example

An illustration of driving duplex formation in a CPR by chemical potential of RNase H in order to detect a target nucleic acid under conditions where there is too little RNase for duplex formation is given below. The chemical potential driving duplex formation is provided by a high concentration of RNase H. The RNAse H enzymatic activity cleaves the probe so as to produce a gapped duplex in which both double stranded regions are more unstable than the original probe: target duplex. Under appropriate experimental conditions this allows the reaction to proceed and cycle even though the reaction is performed at a temperature (65°C) well in excess of the Tm for the probe:target complex.

Reaction Conditions: The following reaction scheme was employed: 1 μL of a 5' ^{32}P labeled probe sequence (the probe is a composite DNA-RNA molecule having a central RNA region disposed between two terminal DNA regions, is 28 bases in length, and is complementary to the target sequence) was added to tubes containing 1 μl of target homologous DNA and 0.5 μl of a buffer composed of 50mM Tris-C1/10mM $MgC1_2$ (pH7.5) and 2.5 μL of ddH_2O. A 1ul aliquot of highly concentrated RNAse H was then added and samples placed at 65°C. The RNAse H concentration was high enough to both facilitate formation of the duplex and to cleave the duplexes formed so as to create a situation where "cycling" occurred driven by the forward chemical potential for enzyme driven duplex formation and the backward potential for product dissociation (e.g., more than one probe could react with the same target). In order to assess whether the chemical potential provided by the enzyme was sufficient to allow detection of 1 target molecule the target concentration (number of molecules) was serially diluted in tenfold increments.

The RNase H digestion procedure for DNA:RNA oligo hybrids was as follows:

1. Probe Labeling: The chimeric "probe" was labeled with kinase and isolated using a 20% denaturing acrylamide gel.

2. Dilutions: The target was serially diluted by a series of 0.1 x dilutions to provide the desired concentrations. The first of the series usually starts at a concentration of 1pmole/µl and is serially diluted to 10^{-12}pmole/µ.

3. Test range: 1µl of diluted target sample was added to a tube. For example, 1µl from the 1pmole/µl tube was placed in the tube marked for the 1pmole of target reaction. 2 µl of sterile ddH_2O was added to the first control tube (-target/-RNase H) to account for the volume contributed by the enzyme and the target in the enzyme and target containing tubes. 1µl sterile ddH_2O was added to the second control tube (-target/+RNase H) to account for the volume contributed by the target in target containing tubes.

4. Reaction cocktail: The following reaction cocktail was made: 0.5µl of 10x RNase H buffer (10X RNase H buffer consists of 0.5M Tris-Cl (pH 7.5) 0.1M MgC12 and should be sterilized) time the number of reactions +1; Xµl of probe (for 20,000 cpm per reaction) times the number of reactions +1; Yµl sterile ddH2O to 3µl times the number of reactions +1; and 3µl total volume times the number of reactions +1.

To test the sensitivity of a probe to detect 1pmole to 10^{-12} pmole of target 13 individual dilutions and 2 controls, -RNase H/-Target and +RNase H/-Target are needed. Therefore, there are 15 reaction in all. The cocktail thus includes:

a. the amount of 10x RNase H reaction buffer with a base volume of 5µl per reaction is: 0.5µl times 16 (the number of reaction +1) which is equal to 8.0µl of 10x RNase H reaction buffer;

b. the amount of probe, with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction, is 0.5µl of probe per reaction times 16 (the number of reactions +1) which is equal to 8.0µl of probe;

c. the amount of ddH_20 needed to make up the volume to 3µl per reaction. Since 0.5µl of 10x RNase H reaction buffer and 0.5µl of probe per reaction, a total of 1µl, is added, an additional 2µl of ddH_20 per reaction (3µl minus volume of probe minus volume of 10x RNase H reaction buffer) times 16 (the number of reactions +1) which is equal to 32µl ddH_20.

5. Distribute Cocktail: Aliquot 3µl of the cocktail into each of the control and target reaction tubes. (This assumes a base volume of 5µl per reaction).

6. Add RNase: Add 1µl of RNase H enzyme to the target sample tubes and the second control (-target/+RNase H). Do not add enzyme to the first control (-target/-RNase H). This step should be done with care since the enzyme is usually in a high concentration of gylcerol and is thus difficult to pipette. The enzyme can be added to the side of the reaction tube and then spun down into the tubes to get the enzyme in or the enzyme can be added directly to the liquid volume already in the tube. Each tube at this point should have 5µl total volume.

7. Incubate: Incubate all reactions @65°C for 30'; centrifuge briefly after reaction time.

8. Analysis: Add 5µl RNA urea dye (RNA urea dye consists of 0.44g urea in 0.5mls of 10mM Tris pH 7.5, 100mM Na2EDTA, 0.01%BPB&XC in 1 ml.) and centrifuge if necessary. The samples can be run on a 20% denaturing polyacrylamide gel for subsequent analysis.

Fig. 2 shows the reaction products of CPR performed as described above. From left to right the lanes show the reaction products of the following: Control 1 (-RNase H/-target); control 2 (+RNase/-Target); target concentration 10^{-5} pMole; target concentration 10^{-6} pMole; target concentration.10^{-7} pMole; target concentration 10^{-8} pMole; target concentration 10^{-9} pMole; target concentration 10^{-10} pMole; target concentration 10^{-11} pMole; target concentration 10^{-12} pMole. The upper band represents the probe and the lower band represents the RNase H cleavage products.

Other embodiments are within the following claims.

What is claimed is:

1. A method for detecting a single-stranded target nucleic acid which comprises:

a. providing a reaction mixture which includes the target nucleic acid, a complementary single-stranded nucleic acid probe, the probe being present in molar excess relative to the target and having the structure

$$[NA_1\text{-}R\text{-}NA_2]_n$$

wherein NA_1 and NA_2 are DNA sequences, wherein R is a scissile nucleic acid linkage, and wherein n is an integer from 1 to 10, and RNase H, the RNase H being present at a chemical potential sufficient to substantially increase the rate of duplex formation over what would be formed in the absence of the RNase H, and allowing target-probe duplex to form;

b. treating the target-probe duplex from step (a) so as to cleave the probe within a predetermined sequence of the scissile nucleic acid linkage and thereby form at least one intact DNA-containing oligonucleotide fragment from the probe, such fragment being, or being treated so as to be, no longer capable of remaining hybridized to the target nucleic acid;

c. repeating the cycle of steps (a) and (b); and

d. detecting the intact DNA-containing fragments so formed and thereby detecting the single-stranded target nucleic acid.

2. The method of claim 1, where said method is performed at a temperature above the Tm of said duplex.

3. The method of claim 1, wherein said temperature is at least x C°, wherein x is 5, 10, 20, 30, 40, 50, 60, 70, or 80, above said Tm.

4. The method of claim 1, wherein the rate of duplex formation is increased by at least y fold, wherein y is 2, 5, 10, 50, 100, 500, 10^3, 10^4, 10^5, 10^6, by the addition of RNase H.

5. The method of claim 1, wherein the rate of duplex formation in the absence of RNase H is substantially zero.

6. The method of claim 1, wherein the concentration, number of molecules of, or the chemical potential of, the RNase H is greater than the concentration, number of molecules present, or chemical potential of said probe, said target, both said probe and said target, said duplex, or the combination of said probe, said target, and said duplex, e.g., at least z fold greater, wherein z is at least 1, 2, 5, 10, 25, 50, 100, 500, 10^3, 10^4, 10^5 or, 10^6.

7. The method of claim 1, wherein the concentration, number of molecules of, or the chemical potential of, the RNase H is sufficient to allow: detection of one target molecule in a biological sample, with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction, detection of a target strand at a concentration of 10^{-5} pMole or less, the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-6} pMole or less, the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-7} pMole or less, the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-8} pMole or less, the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-9} pMole or less, the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-10} pMole or less, the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; detection of a target strand at a concentration of 10^{-11} pMole or less, preferably the detection being carried out with a probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction; or detection of a target strand at a concentration of 10^{-12} pMole or less, the detection being carried out with a CPR probe labeled with an activity of 40,000 cpm/µl, using 20,000 cpm per reaction.

8. The method of claim 1, wherein said probe and said target are present in substantially equal amount, concentration, number, or chemical potentials in said reaction mix.

9. The method of claim 1, wherein the ratio by weight, molarity, number, concentration, or chemical potential of said probe to said target is less than or equal to 1:1, 2:1, 5:1, 10:1, 25:1, 50:1, 100:1, or 10^n:1, wherein n is an integer between 3 and 10, inclusive.

10. The method of claim 1, wherein the ratio by weight, molarity, number, concentration, or chemical potential of the RNase H to the single strand in the highest concentration is greater than 1:1, 2:1, 5:1, 10:1, 25:1, 50:1, 100:1, or 10^n:1, wherein m is an integer between 3 and 10, inclusive.

11. The method of claim 1, wherein said RNase H will accelerate the rate of duplex formation at least n-fold, wherein n is 2, 5, 10, 50, 100, 500, 10^3, 10^4, 10^5, 10^6.

12. The method of claim 1, wherein: the target is a DNA molecule; the target is an RNA molecule; the target is a synthetic, purified natural, genetically engineered, or recombinant DNA or RNA molecule; the target is a naturally occurring nucleic acid, e.g., a genomic molecule, or chromosome, e.g., a viral, bacterial, plant, or animal nucleic acid.

13. A method of claim 1, wherein the oligonucleotide fragment in step (b) is labelled with a detectable marker and labelled fragments are detected in step (d).

14. A method of claim 1, wherein the oligonucleotide fragment is step (b) is unlabelled, but capable of being labeled with a detectable marker, the fragment is so labelled prior to step (c) or (d), and labelled fragments are detected in step (d).

15. A method of claim 1, wherein the scissile nucleic acid linkage is an RNA sequence.

16. A methods of claim 1, wherein NA_1 and NA_2 independently comprise from 0 to about 20 deoxyribonucleotides and R comprises for 1 to about 100 ribonucleotides.

17. A method of claim 1, wherein n is an integer from 2 to 10 and wherein at least one of NA_1 or NA_2 varies within the probe.

18. A method of claim 1, wherein n is 1.

19. A method of claim 1, wherein the treating in step (b) comprises contacting the double-stranded target-probe complex with a double-strand specific ribonucluclease.

20. A method of claim 1, wherein the probe is immobilized on a solid support.

21. A method of claim 15, wherein the unlabelled fragment has a 3'-hydroxyl group and wherein the labelling of the fragment comprises RNA tailing from the 3'-hydroxyl group.

22. A reaction mixture for detection of a single-stranded target nucleic acid, comprising:

a single-stranded target nucleic acid;

a complementary single-stranded nucleic acid probe, the probe being present in molar excess relative to the target and having the structure $[NA_1\text{-}R\text{-}NA_2]_n$ wherein NA_1 and NA_2 are DNA sequences, wherein R is a scissile nucleic acid linkage, and wherein n is an integer from 1 to 10; and

a RNase H, the RNase H being present at a chemical potential sufficient to substantially increase the rate of duplex formation over what would be formed in the absence of the RNase H.

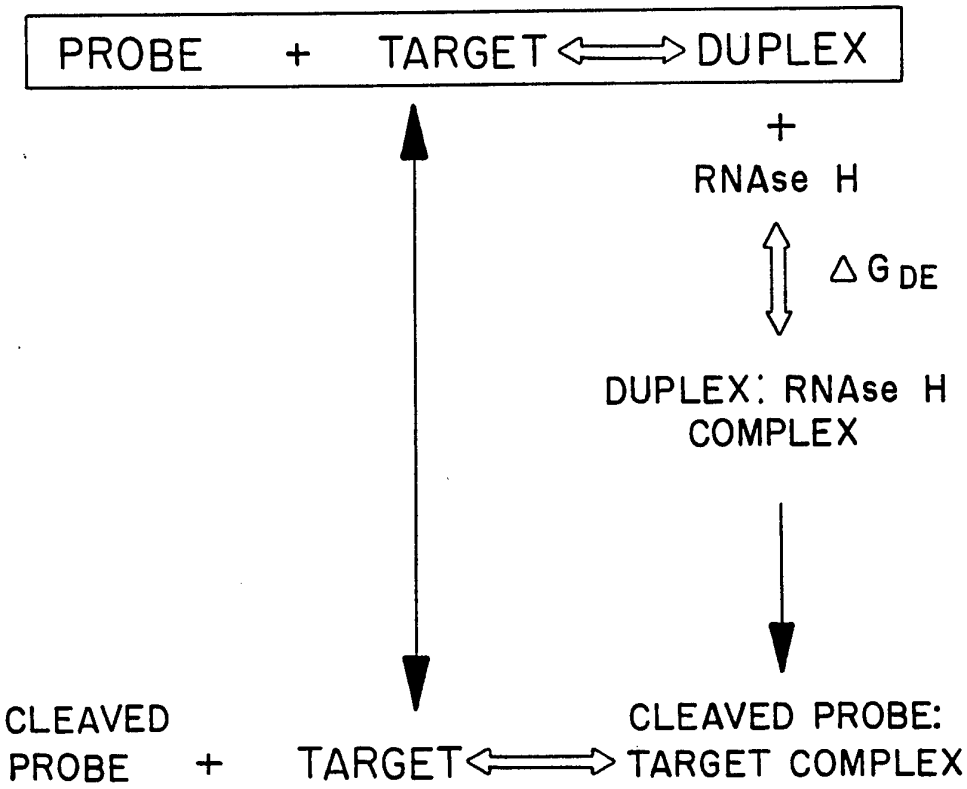
23. The reaction mixture of claim 22, wherein the detection is performed at a temperature above the Tm of said duplex.

24. The reaction mixture of claim 22, wherein the rate of duplex formation is increased by at least n fold, wherein n is 2, 5, 10, 50, 100, 500, 10^3, 10^4, 10^5, 10^6, by the addition of RNase H.

25. The reaction mixture of claim 22, wherein the rate of duplex formation in the absence of RNase H is substantially zero.

26. The reaction mixture of claim 22, wherein the concentration, number of molecules of, or the chemical potential of, the RNase H is greater than the concentration, number of molecules present, or chemical potential of said probe, said target, both said probe and said target, said duplex, or the combination of said probe, said target, and said duplex, e.g., at least n fold greater, wherein n is at least 1, 2, 5, 10, 25, 50, 100, 500, 10^3, 10^4, 10^5 or, 10^6.

FIG. 1


PROBE + TARGET ⟺ DUPLEX
+
RNAse H
ΔG DE
DUPLEX: RNAse H COMPLEX
CLEAVED PROBE + TARGET ⟺ CLEAVED PROBE: TARGET COMPLEX

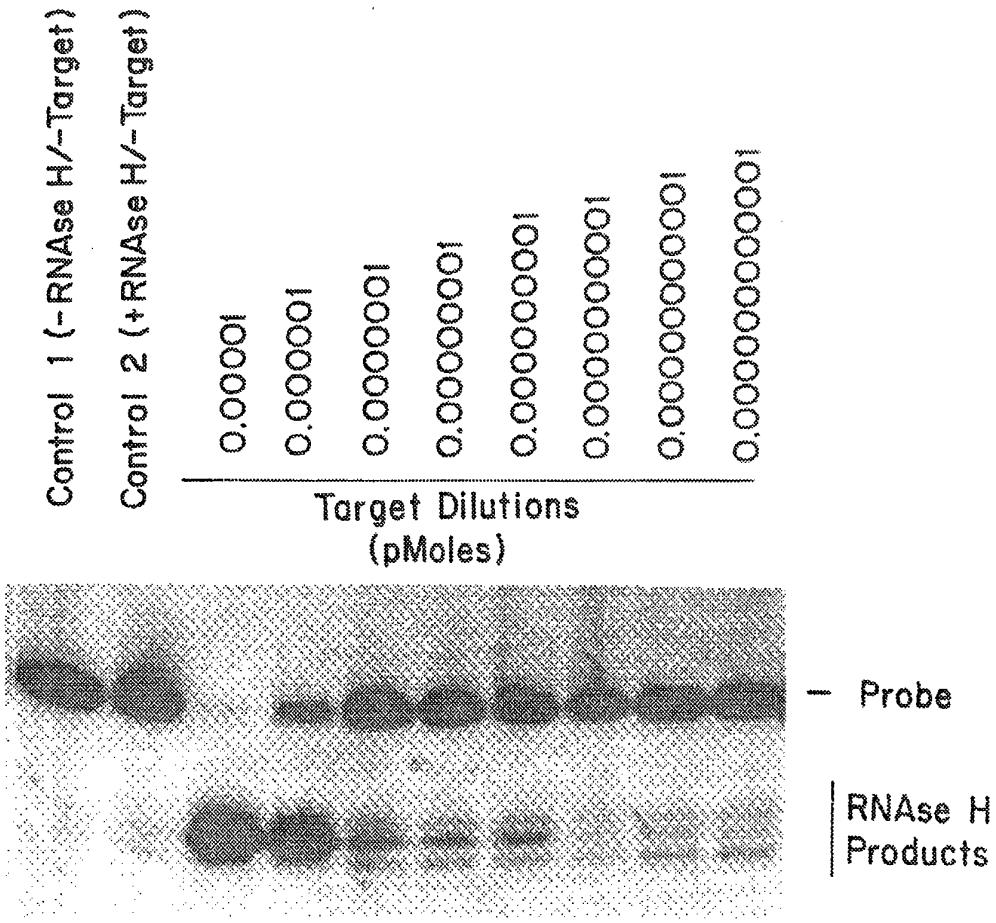

Control 1 (-RNAse H/-Target)
Control 2 (+RNAse H/-Target)
0.00001
0.000001
0.0000001
0.00000001
0.000000001
0.0000000001
0.00000000001
Target Dilutions
(pMoles)
Probe
RNAse H
Products

FIG. 2

INTERNATIONAL SEARCH REPORT

Inter onal application No.
PCT/US94/06855

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) : C12Q 1/68; C12P 19/34
US CL : 435/6, 91.2, 91.53
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2, 91.53

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,011,769 (DUCK ET AL) 30 April 1991, see especially, column 4, lines 17, 18, 22-50 and column 5, lines 14-34.	1, 4, 6, 8-22, 24, and 26 --- 2, 3, 5, 7, 23, and 25
Y	BioTechniques, Volume 9, Number 2, issued 1990, Duck et al, "Probe Amplifier System Based on Chimeric Cycling Oligonucleotides", pages 142-147, see entire document.	2, 3, 5, 7, 23, and 25
Y	Sambrook et al., "Molecular Cloning - A Laboratory Manual", published 1989 by Cold Spring Harbor Laboratory Press (NY), pages 11.31-11.32, see entire document.	2, 3, 5, 7, 23, and 25

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

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

Date of the actual completion of the international search
10 AUGUST 1994

Date of mailing of the international search report
AUG 29 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
MINDY B. FLEISHER
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*