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[54] SEQUENCE-SPECIFIC DETECTION OF NUCLEIC ACID HYBRIDS USING A DNA-BINDING MOLECULE OR ASSEMBLY CAPABLE OF DISCRIMINATING PERFECT HYBRIDS FROM NON-PERFECT HYBRIDS

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[73] Assignee: The Gene Pool, Inc., Seattle, Wash.

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[21] Appl. No.: 353,476

[22] Filed: Dec. 9, 1994

[51] Int. Cl. 6 C12Q 1/70; C12Q 1/68; C07H 21/04

[52] U.S. Cl. 435/5; 435/6; 436/94; 536/24.3

[58] Field of Search 536/24.3, 23.1; 435/5, 6, 810; 436/94

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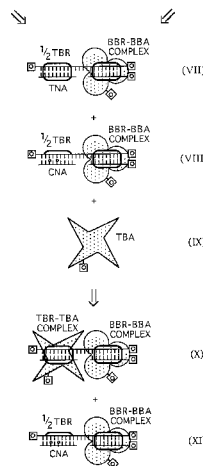
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[57] ABSTRACT

This invention is a novel method for detecting and localizing specific nucleic acid sequences in a sample with a high degree of sensitivity and specificity. The method and novel compositions used in the method involve the use of Probe Nucleic Acids, the production of nucleic acid binding regions and the use of nucleic acid Target Binding Assemblies to detect and localize specific Target Nucleic Acids. The detection and localization of the Target Nucleic Acid is accomplished even in the presence of nucleic acids which have similar sequences. The method provides for a high degree of amplification of the signal produced by each specific binding event. In particular, methods and compositions are presented for the detection of HIV and HPV DNA in samples. These methods and compositions find use in diagnosis of disease, genetic monitoring, forensics, and analysis of nucleic acid mixtures. Some of the novel compositions used in the detection method are useful in preventing or treating pathogenic conditions.

40 Claims, 27 Drawing Sheets



-continued

Thr	Ala	Thr	Pro	Ser	Ala	Leu	Ile	Thr	Thr	Asn	Met	Val	Ala	Met	Glu
		195					200					205			
Ala	Ile	Cys	Pro	Glu	Gly	Ile	Ala	Arg	Leu	Ala	Asn	Ser	Gly	Ile	Asn
	210					215					220				
Val	Met	Gln	Val	Ala	Asp	Leu	Gln	Ser	Ile	Asn	Ile	Ser	Gly	Asn	Gly
225					230					235					240
Phe															

(2) INFORMATION FOR SEQ ID NO:117:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 10 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: NO

(i v) ANTI-SENSE: NO

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:117:

GGGAMTNYCC

1 0

What is claimed is:

1. A method for detecting or localizing specific nucleic acid sequences with a high degree of sensitivity and specificity which comprises:

- (a) adding PNAs containing a $\frac{1}{2}$ BBR and a $\frac{1}{2}$ TBR to a sample containing or suspected of containing TNAs containing $\frac{1}{2}$ TBR sequences, to form a complex having target binding regions, TBRs, formed by the hybridization of complementary $\frac{1}{2}$ TBRs present in the PNAs and TNAs respectively;
- (b) binding the TBRs formed in step (a) to a TBA to form a TBA-TNA-PNA complex, provided that said binding may occur prior to, during or subsequent to said hybridization of step (a);
- (c) adding Booster Nucleic Acids, BNAs, containing booster binding regions, $\frac{1}{2}$ BBRs, to the complex formed in step (b) such that the $\frac{1}{2}$ BBRs in the BNAs hybridize with the $\frac{1}{2}$ BBR sequences present in the PNAs or to $\frac{1}{2}$ BBRs present in BNAs already bound to the PNA, to form BBRs, such that TBA-TNA-PNA-(BNA)_n complexes are formed;
- (d) adding Hairpin Nucleic Acids, HNAs, containing $\frac{1}{2}$ BBR sequences, to the complex formed in step (c) such that the $\frac{1}{2}$ BBRs in the HNAs hybridize with any available $\frac{1}{2}$ BBR sequences present in the BNAs of the complex of step (c), thereby capping the extension of the BNAs onto the TBA-TNA-PNA-(BNA)_n complexes of step (c) to form TBA-TNA-PNA-(BNA)_n-HNA complexes;
- (e) adding Booster Binding Assemblies, BBAs, linked to indicator moieties, to the TBA-TNA-PNA-(BNA)_n-HNA complexes formed in step (d) to form TBA-TNA-PNA-(BNA-BBA)_n-HNA complexes, provided that said BBAs are added prior to, concurrent with or subsequent to addition of said BNAs of step (c); and
- (f) detecting the signals produced by the indicator moieties linked to the TBAs, PNAs, BNAs, BBAs or HNAs in the TBA-TNA-PNA-(BNA-BBA)_n-HNA complexes of step (e);

wherein the TNA comprises:

- (i) one or more specific $\frac{1}{2}$ TBR sequences, the presence or absence of which in a particular sample is to be confirmed;

the PNA comprises:

- (i) a single-stranded sequence, $\frac{1}{2}$ TBR, which is capable of forming, under hybridizing conditions, a hybrid, TBR, with a $\frac{1}{2}$ TBR present in a target nucleic acid (TNA);
- (ii) a single stranded sequence, $\frac{1}{2}$ BBR, which is capable of forming, under hybridizing conditions, a hybrid BBR with a $\frac{1}{2}$ BBR present in a booster nucleic acid (BNA); and

- (iii) an OSA, which is no attached support and/or indicator, or an attached support or indicator or both selected from the group consisting of attachment to beads, polymers, proteins, peptides and surfaces, and/or indicators;

the BNA comprises:

- (i) a $\frac{1}{2}$ BBR, as shown in FIG. 1(IIb), which has a sequence which is complementary to a $\frac{1}{2}$ BBR sequence in a PNA and which is capable of forming, under hybridizing conditions, a hybrid, BBR, with the PNA;
- (ii) an OSA, which is no attached support or indicator, or is an attached support or indicator or both selected from the group consisting of attachment to beads, polymers, proteins, peptides, and surfaces, and/or indicators;
- (iii) additional hybridization sites, $\frac{1}{2}$ BBRs, for other BNAs; and
- (iv) sequences, $\frac{1}{2}$ BBRs, which can hybridize to BNAs already hybridized to the PNA;

the BBA comprises:

- (i) a molecule or assembly, or a portion of a molecule or assembly which is capable of selectively binding to a BBR; and
- (ii) no attached support and/or indicator, or an attached support or indicator or both which is selected from

the group consisting of attachment to beads, polymers, proteins, peptides and surfaces, and/or indicators;

and the TBA comprises:

- (i) a molecule or assembly, or a portion of a molecule or assembly which is capable of selectively binding to a TBR; and
- (ii) no attached support and/or indicator, or an attached support or indicator or both which is selected from the group consisting of attachment to beads, polymers, proteins, peptides and surfaces, and/or indicators.

2. In a solid phase hybridization method for detecting the presence of a target polynucleotide involving: immobilizing a target polynucleotide, if present in a test sample, directly or via an intermediate capture structure, on a solid phase at a capture site; before, during or after said immobilization, attaching a detectable label to said target polynucleotide, if present; and detecting said label, if any, at said capture site; the improvement comprising:

- (a) using a Target Binding Assembly, TBA, as the means for achieving immobilization of said target polynucleotide, wherein said TBA is a nucleic acid binding molecule or assembly which cooperates in and stabilizes the hybridization of the target nucleic acid, TNA, with a specific probe nucleic acid, PNA, thereby forming one or more TBRs, wherein the TBA is capable of binding to and stabilizing the probe-target hybrid in a sequence specific manner, and further wherein the TBA is capable of discriminating between a hybrid formed by the probe and the target nucleic acid, and a hybrid having one or more mismatches formed by the probe and a closely related or unrelated sequence; and
- (b) including in the PNA a single stranded sequence, $\frac{1}{2}$ BBR, capable of binding a Booster Nucleic Acid, BNA, containing a single stranded complementary $\frac{1}{2}$ BBR which, upon hybridization with the $\frac{1}{2}$ BBR in the PNA, forms a BBR capable of binding one or more labeled or unlabeled Booster Binding Assemblies, BBAs.

3. A method for nucleic acid detection comprising

- (a) obtaining a sample containing a hybrid to be detected or containing a target nucleic acid and a probe nucleic acid which hybridize to form a probe-target hybrid; and
- (b) contacting the hybrid of step (a) with a nucleic acid binding molecule or assembly, TBA, wherein the TBA is capable of binding to and stabilizing the probe-target hybrid in a sequence specific manner, and further wherein the TBA is capable of discriminating between a hybrid formed by the probe and the target nucleic acid, and a hybrid having one or more mismatches formed by the probe and a closely related or unrelated sequence.

4. The method of claim 3 which further comprises contacting the hybrid of step (a) or (b) with a label that binds specifically to the nucleic acid bound by the TBA.

5. The method of claim 4 wherein said specific label is a booster nucleic acid which hybridizes with a portion of the probe nucleic acid not involved in hybridization with the target nucleic acid.

6. The method of claim 3 wherein the probe nucleic acid, PNA, comprises:

- (a) a single-stranded sequence, $\frac{1}{2}$ TBR, which is capable of forming, under hybridizing conditions, a hybrid, TBR, with a $\frac{1}{2}$ TBR present in a target nucleic acid (TNA);

- (b) a single stranded sequence, $\frac{1}{2}$ BBR, which is capable of forming, under hybridizing conditions, a hybrid BBR, with a $\frac{1}{2}$ BBR sequence present in a booster nucleic acid (BNA) upon contacting said BNA with said PNA; and

- (c) an OSA, which is no attached support and/or indicator, or an attached support or indicator or both, selected from the group consisting of beads, polymers, proteins, peptides, surfaces, and indicators;

10 wherein said TBR is capable of binding with high affinity to said nucleic acid binding molecule or assembly (TBA) and wherein said BBR is capable of binding with high affinity to a second nucleic acid binding molecule or assembly (BBA), said BBA being a nucleic acid binding molecule or assembly which cooperates in the PNA-BNA or BNA-BNA hybridization, said BBA capable of binding to and stabilizing PNA-BNA or BNA-BNA hybrids in a sequence specific manner, and further wherein the BBA is capable of discriminating between a hybrid formed by the PNA or BNA and a BNA, and a hybrid having one or more mismatches formed by the PNA or BNA and a closely related or unrelated sequence.

7. The method of claim 6 wherein said booster nucleic acid (BNA) comprises:

- (a) a $\frac{1}{2}$ BBR which has a sequence which is complementary to a $\frac{1}{2}$ BBR sequence in a PNA or another BNA and which is capable of forming, under hybridizing conditions, a hybrid, BBR, with the PNA;

- (b) an OSA which is not an attached support or indicator, or is an attached support or indicator selected from the group consisting of beads, polymers, proteins, peptides, surfaces, and indicators; and

- (c) additional hybridization sites, $\frac{1}{2}$ BBRs, for hybridization with additional BNAs so as to form a BNA polymer;

wherein said BBR is capable of binding with high affinity to said BBA.

8. The method of claim 7 wherein a Hairpin Nucleic Acid (HNA) is used to terminate polymerization of BNAs, wherein said HNA under hybridizing conditions is capable of forming a hairpin while at the same time having a single-stranded sequence, $\frac{1}{2}$ BBR, which is capable of binding to a BNA to form a BBR capable of binding a BBA with high affinity.

9. The method of claim 6 wherein said TBR is a nucleic acid binding protein recognition site.

10. The method of claim 9 wherein the TBR is a nucleic acid binding protein recognition site present in the genome of a pathogen or is a binding site associated with a pathogenic condition in a vertebrate genome.

11. The method of claim 9 wherein the TBR is the HIV-LTR or a portion thereof.

12. The method of claim 6 for detecting a specific TNA sequence, comprising the steps of:

- (a) hybridizing said TNA with said PNA to form a TBR;
- (b) hybridizing said PNA with a BNA containing a $\frac{1}{2}$ BBR whose sequence is complementary to a $\frac{1}{2}$ BBR sequence in the PNA;

- (c) contacting the product of step (b) containing a TBR and a BBR, with a TBA which binds to said TBR;

- (d) adding BBAs to the mixture in step (c) wherein said BBA comprises:

- (i) a molecule or assembly or a portion of a molecule or assembly which is capable of selectively binding to a BBR;
- (ii) a detectible indicator; and

(e) detecting signal produced by means of an indicator attached to the BBA, or the OSA attached to the PNA, or by a shift in migration mobility of the TNA upon electrophoretic separation, or by a combination of these means.

13. The method of claim 12 wherein said indicator is a protein, selected from the group consisting of enzymes capable of catalyzing reactions leading to production of colored reaction products; a radionuclide; and colored beads.

14. The method of claim 6 wherein the target binding assembly, TBA, which is a nucleic acid binding molecule or assembly which cooperates in and stabilizes the hybridization of the target nucleic acid, TNA, with a specific probe nucleic acid, PNA, or the booster binding assembly, BBA, which is a nucleic acid binding molecule or assembly which cooperates in and stabilizes the hybridization of the probe nucleic acid, PNA, with a booster nucleic acid, NBA, or the hybridization between a BNA and a BNA, comprises at least one nucleic acid recognition unit, and one or all of the molecules, sequences, or parts thereof selected from the group consisting of linker molecules or sequences, an assembly molecule or sequence, an asymmetry molecule or sequence, a nuclear localization signal sequence (NLS) and an OSA.

15. The method of claim 14 wherein the DNA recognition unit is selected from the group consisting of an NF-kB binding unit, an SP1 binding unit, a TATA binding unit, a human papillomavirus E2 binding unit, an HPV LTR binding unit, and an HIV LTR binding unit.

16. The method of claim 15 wherein the DNA recognition unit has the sequence selected from the group consisting of SEQ ID NO. 63, SEQ ID NO. 64, SEQ ID NO. 65, SEQ ID NO. 66, SEQ ID NO. 67, SEQ ID NO. 68, SEQ ID NO. 69, SEQ ID NO. 70, SEQ ID NO. 71, SEQ ID NO. 72, SEQ ID NO. 73, SEQ ID NO. 74, SEQ ID NO. 75, SEQ ID NO. 76, SEQ ID NO. 77, SEQ ID NO. 78, SEQ ID NO. 79, SEQ ID NO. 80, SEQ ID NO. 81, SEQ ID NO. 82, SEQ ID NO. 83, SEQ ID NO. 84, SEQ ID NO. 93, SEQ ID NO. 94, SEQ ID NO. 95, SEQ ID NO. 96, SEQ ID NO. 97, and SEQ ID NO. 98.

17. The method of claim 14 wherein the linker sequence is an oligopeptide which does not interfere with the DNA recognition function of the DNA recognition unit and which provides stability and control over the spacing of the DNA recognition unit from the remainder of the TBA.

18. The method of claim 17 wherein the linker sequence is an oligopeptide sequence from the interdomain primary sequence of a structural protein.

19. The method of claim 14 wherein the assembly sequence is an oligopeptide sequence which directs the folding and association of DNA recognition units.

20. The method of claim 19 wherein the assembly molecule or sequence is the bacteriophage lambda cro protein or the CI protein, or is a derivative thereof selected from the group consisting of SEQ ID NO. 104, SEQ ID NO. 105, SEQ ID NO. 106, SEQ ID NO. 107, and SEQ ID NO. 108.

21. The method of claim 14 wherein the asymmetry sequence directs the association of DNA recognition and assembly sequences in a predetermined order.

22. The method of claim 21 wherein the asymmetry molecule or sequence is insulin, gonadotropic hormone, FSH, HCG, LH, ACTH, or relaxin or a portion of any of these molecules capable of acting as an asymmetry molecule or sequence.

23. The method of claim 22 wherein the asymmetry sequence is selected from the group consisting of SEQ ID

NO. 85, SEQ ID NO. 86, SEQ ID NO. 87, SEQ ID NO. 88, SEQ ID NO. 89, SEQ ID NO. 90, SEQ ID NO. 91, and SEQ ID NO. 92.

24. The method of claim 14 wherein the NLS is an oligopeptide which directs the migration and uptake of a protein or complex associated with said NLS into the nucleus of a cell.

25. The method of claim 24 wherein the NLS is selected from the group consisting of SEQ ID NO. 72 and SEQ ID NO. 103.

26. The method of claim 14 wherein the TBA is HIV Detect I-IV or HPV Detect I-IV.

27. The method of claim 14 wherein the TBA has a sequence selected from the group consisting of SEQ ID NO. 109, SEQ ID NO. 110, SEQ ID NO. 111, SEQ ID NO. 112, SEQ ID NO. 113, SEQ ID NO. 114, SEQ ID NO. 115, and SEQ ID NO. 116.

28. The method of claim 14 to bind a particular nucleic acid sequence in a target nucleic acid sample which comprises:

(a) fragmenting the nucleic acid in the target nucleic acid sample;

(b) contacting, under hybridizing conditions, the fragmented nucleic acid with a probe nucleic acid complementary to the particular nucleic acid sequence of interest, wherein said probe nucleic acid, upon hybridization with said particular nucleic acid sequence of interest forms a target binding region to which said TBA specifically binds.

29. The method of claim 28 wherein said probe nucleic acid, in addition to sequences complementary to said particular nucleic acid sequence of interest, also has additional sequences to which a booster nucleic acid can bind to form a booster binding site to which a labeled booster binding assembly can bind to provide a signal showing and amplifying the binding of the probe nucleic acid to the target nucleic acid sequence of interest.

30. The method of claim 28 further comprising the step of:

(c) monitoring the shift in mobility of nucleic acids in the target nucleic acid sample as a function of the size such that binding of the TBA to a particular fragment in the sample modifies the mobility of the fragment.

31. A diagnostic or forensic test kit for the detection in a sample of nucleic acid having a specific sequence composition, TNA, which comprises:

(a) a first nucleic acid probe, PNA, complementary to nucleic acid with said specific sequence composition, the presence of which is to be ascertained in a test sample, wherein said first nucleic acid probe, PNA, and said nucleic acid with said specific sequence composition, TNA, form, upon hybridization, a binding site, TBR, for a first nucleic binding molecule, assembly, or protein, TBA, and wherein said first nucleic acid probe, PNA, further comprises additional sequence complementary to a second nucleic acid probe, BNA;

(b) a first nucleic acid binding molecule, assembly, or protein, TBA, specific for the hybrid formed by hybridization of said first nucleic acid probe, PNA, and said nucleic acid with specific sequence composition, TNA, wherein said TBA is capable of binding to and stabilizing the probe-target hybrid in a sequence-specific manner, and further wherein the TBA is capable of discriminating between a hybrid formed by the probe and the target nucleic acid, and a hybrid having one or more mismatches formed by the probe and a closely related or unrelated sequence;

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- (c) a second nucleic acid probe, BNA, complementary to said additional sequence in said first nucleic acid probe, PNA, wherein, upon hybridization of said first and second nucleic acid probes, a binding site, BBR, for a second nucleic acid binding molecule, assembly, or protein, BBA, is formed; and 5
- (d) a second nucleic acid binding molecule, assembly, or protein, BBA, which binds specifically to the hybrid formed upon hybridization of said first nucleic acid probe, PNA, and said second nucleic acid probe, BNA, wherein said second nucleic acid binding molecule, assembly, or protein, BBA, is labeled with a detectable label. 10

32. The diagnostic or forensic test kit of claim **31** wherein said first nucleic acid probe is complementary to the HIV LTR, such that upon hybridization of said first nucleic acid probe with an HIV LTR, a binding site is formed for NF-kB or a subunit thereof, SP1, TATA binding protein, HIV-Detect I, II, III, or IV, or HIV-Lock. 15

33. The diagnostic or forensic test kit of claim **32** wherein said first DNA binding protein is NF-kB or a subunit thereof, SP1, TATA binding protein, HIV-Detect I, II, III, or IV, or HIV-Lock. 20

34. The diagnostic or forensic test kit of claim **33** wherein said first nucleic acid probe, in addition to being complementary to the HIV LTR, comprises a sequence encoding the bacteriophage lambda left or right operator and said second nucleic acid probe comprises sequences complementary to said bacteriophage lambda left or right operator sequences in said first nucleic acid probe, such that upon hybridization of said first and second nucleic acid probes, a binding site for the bacteriophage lambda CI repressor protein, the bacteriophage lambda cro protein or a derivative or homolog thereof, is formed. 25 30

35. The diagnostic or forensic test kit of claim **34** wherein said second DNA binding protein is the bacteriophage lambda CI repressor protein, the bacteriophage lambda cro protein or a derivative or homolog thereof. 35

36. A method of differentially binding a nucleic acid binding molecule, assembly, or protein, TBA, to a nucleic acid sequence correlated with a pathogenic condition which comprises: 40

- (a) selecting a particular configuration of nucleic acid binding molecule, assembly, or protein sequences, TNA, present in the nucleic acid sequence correlated with a pathogenic condition as a target sequence for 45

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designing a probe nucleic acid, PNA, which will hybridize to said TNA if present in a test sample, and further, ensuring that a binding site for an available nucleic acid binding molecule, assembly, or protein, TBA, is formed upon hybridization of said probe nucleic acid and said TNA;

- (b) selecting a TBA as a nucleic acid binding molecule, assembly, or protein which specifically binds to the selected TNA, but which does not bind to sequences not correlated with said pathogenic condition, and provided that said TBA is capable of binding to and stabilizing the probe-target hybrid in a sequence specific manner, and further wherein the TBA is capable of discriminating between a hybrid formed by the probe and the target nucleic acid, and a hybrid having one or more mismatches formed by the probe and a closely related or unrelated sequence;
- (c) hybridizing said PNA with a test sample suspected of containing said TNA;
- (d) contacting said TBA with any TBR hybrids formed in step (b); and
- (e) detecting any binding of said TBA with said TBR hybrids.

37. The method of claim **4** comprising amplifying signal obtained through binding the PNA to the TNA which comprises binding BNAs to the PNA-TNA hybrid and binding labeled BBAs to the BBRs formed between the BNA and PNA and between successive BNAs.

38. The method of claim **14** which further comprises assembling a nucleic acid binding complex, TBA or BBA, which comprises using asymmetry sequences to direct the association or non-association of components of the nucleic acid binding complex.

39. The method of claim **14** which further comprises assembling a nucleic acid binding complex, TBA or BBA, which comprises using assembly sequences derived from bacteriophage lambda cro or CI to act as or assemble associated components of the nucleic acid binding complex.

40. The method of claim **14** wherein said TBA, said BBA, or both said TBA and said BBA is a multimeric molecule or assembly prepared by linking assembly, asymmetry, or piloting molecules, sequences, or subunits to be incorporated into said multimeric TBA or BBA, and recovering said multimeric TBA or BBA.

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