



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

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<b>(21) International Application Number:</b> PCT/US90/02049  <b>(22) International Filing Date:</b> 16 April 1990 (16.04.90)  <b>(30) Priority data:</b> 340,031                      18 April 1989 (18.04.89)                      US  <b>(71) Applicant:</b> CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).  <b>(72) Inventors:</b> URDEA, Michael, S. ; 100 Bunce Meadow Road, Alamo, CA 94507 (US). WARNER, Brian ; 1034 Alhambra Avenue, Martinez, CA 94553 (US). RUNNING, Joyce, A. ; 3141 Fitzpatrick Drive, Concord, CA 94519 (US). KOLBERG, Janice, A. ; 179 Schooner Court, Richmond, CA 94804 (US). CLYNE, Jennifer, M. ; 1034 Alhambra Avenue, Martinez, CA 94553 (US). SANCHEZ-PESCADOR, Ray ; 787 Woodland Avenue, San Leandro, CA 94577 (US). HORN, Thomas ; 1583-A Arch Street, Berkeley, CA 94708 (US).		<b>(74) Agents:</b> CIOTTI, Thomas, E. et al.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> IMPROVED AMPLIFIED NUCLEIC ACID HYBRIDIZATION ASSAYS FOR HBV		
<b>(57) Abstract</b>  Amplified solution-phase sandwich nucleic acid hybridization assays for HBV in which (1) analyte is hybridized in solution with sets of amplifier probe oligonucleotides and capture probe oligonucleotides each having a first segment that is complementary to a consensus HBV ds region sequence based on a multiplicity of HBV subtypes and a second segment that is complementary to a unit of an oligonucleotide multimer and an oligonucleotide bound to a solid phase, respectively (2) the resulting product is reacted with the oligonucleotide bound to a solid phase (3), the resulting product is washed to remove unbound materials (4), the bound materials are reacted with the multimer (5), the bound materials are washed to remove unbound multimer (6), the bound materials are reacted with a labeled probe complementary to the oligonucleotide units of the multimer.		

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IMPROVED AMPLIFIED NUCLEIC ACID  
HYBRIDIZATION ASSAYS FOR HBV

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Description

Technical Field

This invention is in the fields of nucleic acid  
15 chemistry and biochemical assays. More particularly, it  
relates to novel nucleic acid multimers and nucleic acid  
hybridization assays.

Background Art

20 Nucleic acid hybridizations are now commonly  
used in genetic research, biomedical research and clinical  
diagnostics. In the basic nucleic acid hybridization as-  
say, single-stranded analyte nucleic acid (either DNA or  
RNA) is hybridized to a labeled nucleic acid probe, and  
25 resulting labeled duplexes are detected. Both radioactive  
and nonradioactive labels have been used.

Variations of this basic scheme have been devel-  
oped to facilitate separation of the duplexes to be  
detected from extraneous materials and/or to amplify the  
30 signal that is detected.

EPA Publication No. 0225807 describes a  
solution-phase nucleic acid hybridization assay in which  
the analyte nucleic acid is hybridized to a labeling probe  
set and to a capturing probe set. The probe-analyte  
35 complex is coupled by hybridization with a solid-supported  
capture probe that is complementary to the capture probe

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set. This permits the analyte nucleic acid to be removed from solution as a solid phase complex. Having the analyte in the form of a solid phase complex facilitates subsequent separation steps in the assay. The labeling probe set is complementary to a labeled probe that is bound through hybridization to the solid phase/analyte complex.

PCT Application 84/03520 and EPA 124221 describe a DNA hybridization assay in which: (1) analyte is annealed to a single-stranded DNA probe that has a tail that is complementary to an enzyme-labeled oligonucleotide, and (2) the resulting tailed duplex is hybridized to an enzyme-labeled oligonucleotide. The Enzo Biochem "Bio-Bridge" labeling system appears to be similar to the system described in these two patent applications. The "Bio-Bridge" system uses terminal deoxynucleotide transferase to add unmodified 3'-polyT-tails to a DNA probe. The polyT-tailed probe is hybridized to the target DNA sequence and then to a biotin-modified polyA.

EPA 204510 describes a DNA hybridization assay in which analyte DNA is contacted with a probe that has a tail, such as a polyT-tail, an amplifier strand that has a sequence, e.g., a polyA sequence, that hybridizes to the tail of the probe and is capable of binding a plurality of labeled strands.

EPA 0317077 published 24 May 1989 (after the priority of the present application) describes a hybridization assay that employs oligonucleotide multimers to achieve amplification. These multimers were used in the above-described solution-phase hybridization assay of EPA Publication No. 0225807. They comprise an oligonucleotide unit that hybridizes to the labeling probe and a multiplicity of other oligonucleotide units that bind to the labeled probe. Assays for a variety of analytes, including hepatitis B virus (HBV) nucleic acid, are described in EPA 0317077. The HBV assay exemplified

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in EPA 0317077 employed sets of capture and labeling probes derived from a single HBV subtype.

The present invention provides an improvement of the HBV assay exemplified in EPA 0317077 which gives fewer  
5 false negatives.

#### Disclosure of the Invention

One aspect of the invention is a solution sandwich DNA hybridization for detecting HBV DNA in an  
10 analyte comprising:

(a) contacting the analyte under hybridizing conditions with an excess of i) a set of amplifier probe oligonucleotides each comprising a first segment having a nucleotide sequence complementary to a segment of the HBV  
15 genome and a second segment having a nucleotide sequence complementary to an oligonucleotide unit of a nucleic acid multimer and (ii) a set of capture probe oligonucleotides each comprising a first segment having a nucleotide sequence complementary to a segment of the HBV genome and  
20 a second segment having a nucleotide sequence complementary to an oligonucleotide bound to a solid phase;

(b) contacting under hybridizing conditions the product of step (a) with the oligonucleotide bound to the  
25 solid phase;

(c) thereafter separating materials not bound to the solid phase;

(d) contacting under hybridizing conditions the solid phase complex product of step (c) with the nucleic  
30 acid multimer, said multimer comprising (i) at least one oligonucleotide unit that is complementary to the second segments of the amplifier probe oligonucleotides and (ii) a multiplicity of second oligonucleotide units that are complementary to a labeled oligonucleotide;

35 (e) removing unbound multimer;

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(f) contacting under hybridizing conditions the solid phase complex product of step (e) with the labeled oligonucleotide;

(g) removing unbound labeled oligonucleotide;  
5 and

(h) detecting the presence of label in the solid phase complex product of step (g), characterized in that the first segments of the amplifier probe oligonucleotides and capture probe oligonucleotides have sequences that are complementary to consensus HBV ds region sequences based on a multiplicity of HBV subtypes.  
10

Another aspect of the invention is a synthetic oligonucleotide useful as an amplifier probe in a sandwich hybridization assay for HBV selected from the group consisting of synthetic oligonucleotides which include the following sequence:  
15

TGACTG (CG) CGATTGGT (GA) GAGGCAGG (AC) GGAGG,  
CTTG (AT) (CT) GGG (GA) TTGAAGTCCCAATCTGGATT,  
20 GTTGCCTCAGCAAACACTTGGCA (CG) AGACC (AT),  
TAAGTTGGCGAGAAAGT (GA) AAAGCCTG (TC) TT (AC),  
GCAGCAA (GA) CCCAAAAGACCCACAA (TG) (TA) C (TG) (TC),  
ATGTATACCCA (GA) AGACA (AG) AAGAAAATTGGT,  
TAGAGGACAAACGGGCAACATACCTTG (AG) TA,  
25 GATGAGGCATAGCAGCAGGATGAAGAGGAA,  
GATAAAACGCCGACACATCCAGCGATA,  
GGACAA (AG) TTGGAGGACA (GA) GAGGTTGGTGAG,  
TTGGAGGTTGGGGACTGCGAATTTTGGCCA,  
CCACCACGAGTCTAGACTCTG (CT) GGTATTGT,  
30 GATTCTTGTCACAAGAAAAACCCCGCCTG,  
CACGAG (CA) AGGGTCCTAGGAATCCTGATGT,  
CAGGGTTTACTGTTCC (TG) GAACTGGAGCCAC,  
CAGGGTCCCCAGTCCTCG (AC) G (AG) AGATTGACG,  
CCGTTGCCGAGCAACGGGGTAAAGGTT (CA) A (GT),  
35 GGTTGCCTCAGCAAACACTTGGCA (GC) AGACC,

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AGTTCCGCAGTATGGATCGGCAGA (CG) GAGCC,  
 CCAGACC (TG) (CG) CTGCGAGCAAAACAAGC (TG) GCT,  
 CAGTTGGCAG (CT) ACA (CG) CCTAGCAGCCATGGA,  
 GGGACGTA (AG) ACAAAGGACGTCCCGC (AC) AGG,  
 5 CGAGA (ACG) GGGTCGTCCGC (AG) GGATTCAGCGCC,  
 CCGCGTAAAGAGAGGTGCGCCCCGTGGTTCG,  
 ACACGG (TA) CCGGCAGATGAGAAGGCACAGAC,  
 C (TG) CCATGC (AGT) ACGTGCAGAGGTGAAGCG,  
 CAAGAGTCCTCTT (AG) TGTAAGACCTTGGGCA,  
 10 AACA (AC) ACAGTCTTTGAAGTA (TG) GCCTCAAGG,  
 CTAATCTCCTCCCCCA (AG) CTCCTCCCAGTC (CT),  
 TGCCTACAGCCTCCTA (AG) TACAAAGA (CT) C (AT),  
 GACATG (AT) ACA (AT) GAGATGATTAGGCAGAGG (GT),  
 CTTTATA (CA) GG (AG) TC (GA) ATGTCCATGCCCAAA,  
 15 AAAA (AC) GAGAGTAACTCCACAG (AT) (AT) GCTCCAA,  
 AGGAGTGCGAATCCCACTCC (AG) AAAGA (GCT) AC,  
 TAA (GA) GATAGGGCATTGTTGGTGGTCT (AG) TA (GA) GC,  
 TCGTCTAACAACAGTAGT (CT) TCCGGAAGTGT,  
 GCTGTAG (CA) TCTTGTTCCTCAAGAATATGGTG, and  
 20 (TC) GCCCTGAGCCTG (AC) GGGCTCCACCCCAAAA, wherein  
 the parentheses indicate two-fold degenerative positions.

Another aspect of the invention is a set of  
 synthetic oligonucleotides useful as amplifier probes in a  
 sandwich hybridization assay for hepatitis B virus  
 25 comprising oligonucleotides which include the following  
 sequences:

TGA CTG (CG) CGATTGGT (GA) GAGGCAGG (AC) GGAGG,  
 CTTG (AT) (CT) GGG (GA) TTGAAGTCCCAATCTGGATT,  
 30 GTTGCCTCAGCAAACACTTGGCA (CG) AGACC (AT),  
 TAAGTTGGCGAGAAAGT (GA) AAAGCCTG (TC) TT (AC),  
 GCAGCAAA (GA) CCCAAAAGACCCACAA (TG) (TA) C (TG) (TC),  
 ATGTATACCCA (GA) AGACA (AG) AAGAAAATTGGT,  
 TAGAGGACAAACGGGCAACATACCTTG (AG) TA,  
 35 GATGAGGCATAGCAGCAGGATGAAGAGGAA,

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GATAAAACGCCGCAGACACATCCAGCGATA,  
 GGACAA (AG) TTGGAGGACA (GA) GAGGTTGGTGAG,  
 TTGGAGGTTGGGGACTGCGAATTTTGGCCA,  
 CCACCACGAGTCTAGACTCTG (CT) GGTATTGT,  
 5 GATTCTTGTCACAACAAGAAAAACCCCGCCTG,  
 CACGAG (CA) AGGGGTCCTAGGAATCCTGATGT,  
 CAGGGTTTACTGTTCC (TG) GAACTGGAGCCAC,  
 CAGGGTCCCCAGTCCTCG (AC) G (AG) AGATTGACG,  
 CCGTTGCCGAGCAACGGGGTAAAGGTT (CA) A (GT) ,  
 10 GGTTGCGTCAGCAAACACTTGGCA (GC) AGACC,  
 AGTTCCGCAGTATGGATCGGCAGA (CG) GAGCC,  
 CCAGACC (TG) (CG) CTGCGAGCAAACAAGC (TG) GCT,  
 CAGTTGGCAG (CT) ACA (CG) CCTAGCAGCCATGGA,  
 GGGACGTA (AG) ACAAAGGACGTCCCGCG (AC) AGG,  
 15 CGAGA (ACG) GGGTCGTCCGC (AG) GGATTCAGCGCC,  
 CCGCGTAAAGAGAGGTGCGCCCCGTGGTCG,  
 ACACGG (TA) CCGGCAGATGAGAAGGCACAGAC,  
 C (TG) CCATGC (AGT) ACGTGCAGAGGTGAAGCG,  
 CAAGAGTCCTCTT (AG) TGTAAGACCTTGGGCA,  
 20 AACA (AC) ACAGTCTTTGAAGTA (TG) GCCTCAAGG,  
 CTAATCTCCTCCCCCA (AG) CTCCTCCAGTC (CT) ,  
 TGCCTACAGCCTCCTA (AG) TACAAAGA (CT) C (AT) ,  
 GACATG (AT) ACA (AT) GAGATGATTAGGCAGAGG (GT) ,  
 CTTTATA (CA) GG (AG) TC (GA) ATGTCCATGCCCAAA,  
 25 AAAA (AC) GAGAGTAACTCCACAG (AT) (AT) GCTCCAA,  
 AGGAGTGCGAATCCACACTCC (AG) AAAGA (GCT) AC,  
 TAA (GA) GATAGGGGCATTTGGTGGTCT (AG) TA (GA) GC,  
 TCGTCTAACAACAGTAGT (CT) TCCGGAAGTGT,  
 GCTGTAG (CA) TCTTGTTCCCAAGAATATGGTG, and  
 30 (TC) GCCCTGAGCCTG (AC) GGGCTCCACCCCAAAA, wherein  
 the parentheses indicate two-fold degenerative positions.

Another aspect of the invention is a synthetic  
 oligonucleotide useful as a capture probe on a sandwich  
 hybridization assay for hepatitis B virus (HBV) selected  
 35 from the group consisting of synthetic oligonucleotides  
 which include the following sequence:



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CTTGGCCCCCAATACCACATCATCCATATA,  
 GAAAGCCAAACAGTGGGGGAAAGCCCTACG,  
 CACTGAACAAATGGCACTAGTAAACTGAGC,  
 5 GAGAAACGG (AG) CTGAGGCCC (AC) CTCCCATAGG,  
 (GC) CGAAAGCCCAGGA (CT) GATGGGATGGGAATA,  
 CGAGGCGAGGGAGTTCTTCTTCTAGGGGAC,  
 TCTTCTGCGACGCGGCGAT (GT) GAGA (TC) CT (GT) CGT,  
 GG (AG) ATACTAACATTGAGATTCCCGAGATTG,  
 10 AGCCC (CA) GTAAAGTT (TC) CC (CG) ACCTTATGAGTC, and  
 CCCAAGGCACAGCTTGGAGGCTTGAACAG, wherein the  
 parentheses indicate two-fold degenerative positions.

Another aspect of the invention is a set of  
 synthetic oligonucleotides useful as capture probes in a  
 15 sandwich hybridization assay for hepatitis B virus  
 comprising oligonucleotides which include the following  
 sequences:

CTTGGCCCCCAATACCACATCATCCATATA,  
 20 GAAAGCCAAACAGTGGGGGAAAGCCCTACG,  
 CACTGAACAAATGGCACTAGTAAACTGAGC,  
 GAGAAACGG (AG) CTGAGGCCC (AC) CTCCCATAGG,  
 (GC) CGAAAGCCCAGGA (CT) GATGGGATGGGAATA,  
 CGAGGCGAGGGAGTTCTTCTTCTAGGGGAC,  
 25 TCTTCTGCGACGCGGCGAT (GT) GAGA (TC) CT (GT) CGT,  
 GG (AG) ATACTAACATTGAGATTCCCGAGATTG,  
 AGCCC (CA) GTAAAGTT (TC) CC (CG) ACCTTATGAGTC, and  
 CCCAAGGCACAGCTTGGAGGCTTGAACAG, wherein the  
 parentheses indicate two-fold degenerative positions.

30

#### Brief Description of the Drawings

FIG. 1 is a schematic representation of the  
 process for the enzymatic preparation of a linear nucleic  
 acid multimer that is described in Example 1.

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FIGS. 2A and 2B are schematic representations of  
 the processes for the chemical preparation of linear and

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branched nucleic acid multimers that are described in Example 2.

FIG. 3 (Parts A-F), illustrate procedures used in making multimers having "comb-like" and/or bifurcated structures.

FIG. 4 is a schematic representation of the sandwich hybridization assay described in Example 3.

FIG. 5 is an autoradiogram showing the results of the dot blot screening tests described in the examples.

FIGS. 6-8 are bar graphs depicting the results of the tests on authentic HBV DNA samples that are described in Example 3B.

FIG. 9 shows partial nucleotide sequences of the capture and amplifier probes used in the HBV assay described in Example 4.

### Modes for Carrying Out the Invention

#### Description of Multimers

The nucleic acid multimers of the invention are linear or branched polymers of the same repeating single-stranded oligonucleotide unit or different single-stranded oligonucleotide units. At least one of the units has a sequence, length, and composition that permits it to bind specifically to a segment of the HBV amplifier probes of the invention. In order to achieve such specificity and stability, this unit will normally be 15 to 50, preferably 15 to 30, nucleotides in length and have a GC content in the range of 40% to 60%. In addition to such unit(s), the multimer includes a multiplicity of units that are capable of hybridizing specifically and stably to a second single-stranded nucleotide of interest, typically a labeled oligonucleotide or another multimer. These units will also normally be 15 to 50, preferably 15 to 30, nucleotides in length and have a GC content in the range of 40% to 60%. When a multimer is designed to be

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hybridized to another multimer, the first and second oligonucleotide units are heterogeneous (different).

The total number of oligonucleotide units in the multimer will usually be in the range of 3 to 50, more usually 10 to 20. In multimers in which the unit that hybridizes to the amplifier is different from the unit that hybridizes to the labeled oligonucleotide, the number ratio of the latter to the former will usually be 2:1 to 30:1, more usually 5:1 to 20:1, and preferably 10:1 to 15:1.

The oligonucleotide units of the multimer may be covalently linked directly to each other through phosphodiester bonds or through interposed linking agents such as nucleic acid, amino acid, carbohydrate or polyol bridges, or through other cross-linking agents that are capable of cross-linking nucleic acid or modified nucleic acid strands. The site(s) of linkage may be at the ends of the unit (in either normal 3'-5' orientation or randomly oriented) and/or at one or more internal nucleotides in the strand. In linear multimers the individual units are linked end-to-end to form a linear polymer. In one type of branched multimer three or more oligonucleotide units emanate from a point of origin to form a branched structure. The point of origin may be another oligonucleotide unit or a multifunctional molecule to which at least three units can be covalently bound. In another type, there is an oligonucleotide unit backbone with one or more pendant oligonucleotide units. These latter-type multimers are "fork-like", "comb-like" or combination "fork-" and "comb-like" in structure. The pendant units will normally depend from a modified nucleotide or other organic moiety having appropriate functional groups to which oligonucleotides may be conjugated or otherwise attached. The multimer may be totally linear, totally branched, or a combination of linear and branched portions. Preferably there will be at

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least two branch points in the multimer, more preferably at least 3, preferably 5 to 10. The multimer may include one or more segments of double-stranded sequences.

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#### Synthesis of Multimers

The multimers may be prepared by cloning (if linear), enzymatic assembly, chemical cross-linking techniques, direct chemical synthesis or a combination thereof. In the case of linear multimers prepared by cloning, nucleic acid sequences that encode the entire multimer or fragments thereof can be made in single- or double-stranded form by conventional cloning procedures. When made in double-stranded form, the multimers/fragments are ultimately denatured to provide single-stranded multimers/fragments. Multimers may be cloned in single-stranded form using conventional single-stranded phage vectors such as M13. Fragments can be linked enzymatically or chemically to form the multimer. When assembled enzymatically, the individual units are ligated with a ligase such as T4 DNA or RNA ligase, as the case may be. When prepared by chemical cross-linking, the individual units may be synthesized with one or more nucleic acids that have been derivatized to have functional groups that provide linking sites or derivatized after the oligonucleotide has been synthesized to provide such sites. A preferred procedure for chemical cross-linking is to incorporate N<sup>4</sup>-modified cytosine bases into the nucleotide as described in EPA Publication No. 0225807.

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When prepared by direct chemical synthesis oligonucleotides containing derivatized nucleic acids or equivalent multifunctional molecules whose functional groups are blocked are made by conventional oligonucleotide synthesis techniques. The functional

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groups are unblocked and oligonucleotide units are synthesized out from the unblocked site(s).

A generic structure for the molecules used to generate branch points in the multimers is as follows:

5

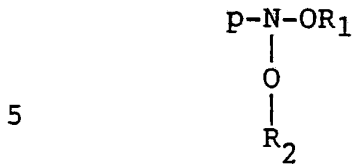


10 where R is an organic moiety, preferably a nucleic acid, R<sup>1</sup> is a hydroxyl protecting group that can be removed under conditions that do not remove synthetic nucleic acid from a solid phase and do not remove exocyclic nitrogen or phosphate protecting groups, X is a phosphorus-containing  
15 group that facilitates nucleic acid synthesis, such as a protected phosphoramidite, phosphonate or phosphate group, Y is a radical derived from a nucleophilic group such as an amino, hydroxyl, sulfhydryl or protected phosphate, and  
20 R<sup>2</sup> is R<sup>1</sup> or a blocking or protective group that can be removed and replaced with hydrogen without affecting R<sup>1</sup>. In molecules used to generate bifurcated or "fork-like" branching, R<sup>1</sup> and R<sup>2</sup> are the same; whereas in molecules used to generate "comb-like" branching, R<sup>2</sup> is a blocking group that is stable in the presence of an R<sup>1</sup> deblocking  
25 reagent. FIG. 3 schematically illustrates the procedures used to synthesize multimers having "comb-like" branches, "fork-like" branches, or combinations thereof.

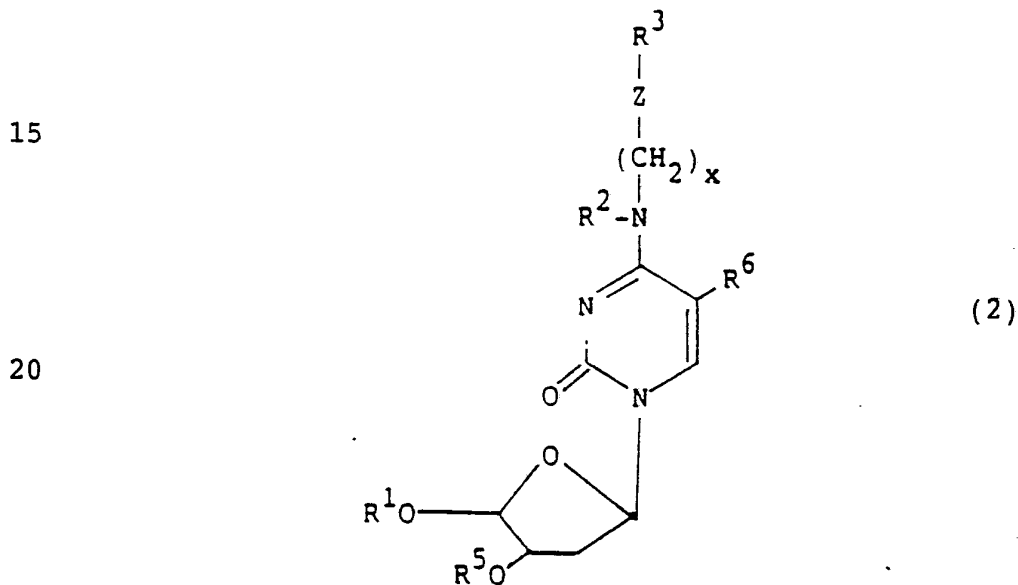
Part A of FIG. 3 depicts a conventional oligonucleotide synthesis scheme for preparing a linear  
30 oligonucleotide, such as the automated phosphoramidite method (Warner et al., DNA (1984) 3:401). The dark block represents a solid support, N represents a nucleotide and p-N-OR<sub>1</sub> (R<sub>1</sub> is equivalent to R<sup>1</sup> below), a conventional nucleotide derivative having appropriate protecting  
35 groups.

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Part B shows the procedure for making a comb-like multimer. The compound

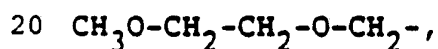
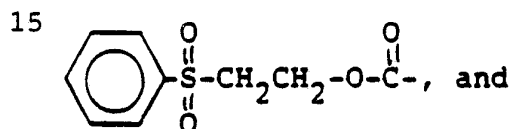
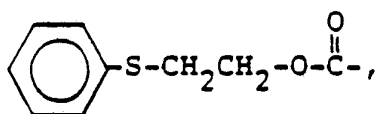
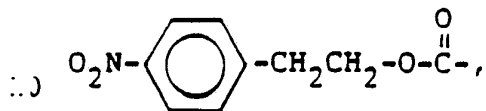
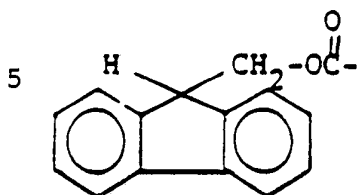
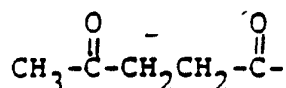


represents a modified base of formula (2) below. An oligomer unit of desired size and sequence is synthesized and left on the support. One or more N<sup>4</sup>-modified cytosine  
10 bases are then incorporated into the chain by said automated procedure. Preferably, the modified base has the formula



where Z is a nucleophile such as -O-, -NH-, -S-, PO<sub>4</sub>=, and  
O  
-OC-O-, R<sup>1</sup> is a blocking or protective group such as  
dimethoxytrityl (DMT) or pixyl that is generally base-  
30 stable and acid sensitive, R<sup>2</sup> is hydrogen or methyl, R<sup>3</sup> is  
a blocking or protective group that can be removed and  
replaced with hydrogen without affecting R<sup>1</sup> such as

-13-



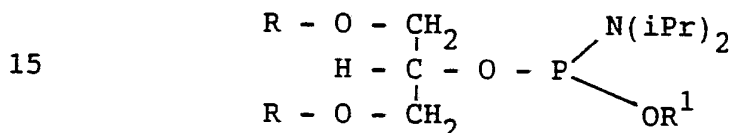
$\text{R}^5$  is a phosphoramidite or other phosphorus derivative that enables addition of nucleotides to the 5' position of an oligonucleotide chain during chemical synthesis (e.g., a phosphodiester, phosphotriester, etc.),  $\text{R}^6$  is methyl, hydrogen, I, Br, or F, and X is an integer in the range of 1 to 8, inclusive. When more than one modified base is incorporated they are preferably spaced by intermediate bases in the chain, most preferably by a -TT- dimer. Additional oligonucleotide units may be incorporated into the backbone followed by additional modified bases and so forth.

The  $\text{N}^4$  nucleophile group is then deprotected ( $\text{R}^3$  is removed) and additional oligonucleotide units are

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generated therefrom by the automated procedure. Residual  $R^1$  groups at the chain termini are removed and the branched "comb-like" multimer is cleaved from the support.

Part C of FIG. 3 depicts the general procedure for making "fork-like" multimers. Again, an oligomer unit of desired size and sequence is synthesized by conventional techniques and left on the support. A blocked, bifunctional phosphorus-containing group (represented as XP in Part C) such as a blocked phosphoramidite, is then incorporated into the chain by the automated procedure. Preferred bifunctional phosphorus-containing groups are blocked phosphoramidites of the formula



where R is said hydroxyl protecting group, iPr is isopropyl, and  $R^1$  is methyl or beta-cyanoethyl. Most preferably R is DMT and  $R^1$  is beta-cyanoethyl.

Alternatively, the  $N^4$ -modified cytosine base where  $R_1=R_2$  (e.g., DMT) can be used.

The two protecting groups are then removed and additional oligonucleotide units are generated therefrom by the automated procedure. Residual  $R^1$  groups are removed and the bifurcated multimer is cleaved from the support.

Parts D and E depict procedures where two or more bifurcated multimers, "comb-like" multimers or combinations thereof are spliced together enzymatically or chemically. Generally, the bifurcated and/or "comb-like" multimers are prepared as above and removed from the support. They are then combined in solution using the enzymatic or chemical linkage procedures described above.

Part F shows the procedure for synthesizing a multiple "comb-like" multimer. This procedure is a variation of the procedure shown in Part B and involves in-

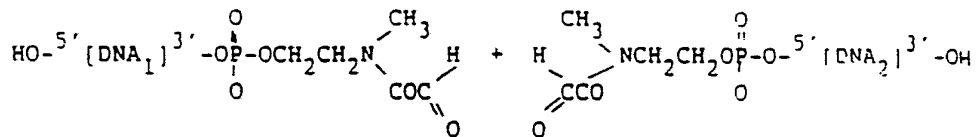




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where DNA<sub>1</sub> and DNA<sub>2</sub> represent DNA subfragments which may be the same or different. Reaction of this fragment with sodium periodate cleaves the fragment into the following subfragments:

5



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Alternatively, the 1,2-diol group may be replaced with linker groups that contain a hydroxylamine-sensitive linkage, a base-sensitive sulfone linkage, or a thiol-sensitive disulfide linkage. Such linker groups may be derived from conventional cross-linking agents that are used to conjugate proteins to other entities. Likewise, protecting groups other than DMT may be used.

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#### Hybridization Assay

The solution-phase sandwich hybridization assay for HBV is carried out as follows. Single-stranded analyte nucleic acid is incubated under hybridization conditions with an excess of two single-stranded nucleic acid probe sets, (1) a capture probe set having first binding sequences complementary to consensus HBV ds region sequences based on a multiplicity of HBV subtypes and a second binding sequence that is complementary to a single-stranded oligonucleotide bound to a solid phase, and (2) an amplifier probe set having first binding sequences that are complementary to the consensus HBV ds region sequences based on a multiplicity of HBV subtypes and a second binding sequence that is complementary to an oligonucleotide unit of the amplification multimer. The resulting product is a three component nucleic acid complex of the two probes hybridized to the analyte by their first binding sequences. The second binding

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sequences of the probes remain as single-stranded tails as they are not complementary to the analyte. Multiple probes of each type may be used.

This complex is then added under hybridizing conditions to a solid phase having a single-stranded oligonucleotide bound to it that is complementary to the second binding sequence of the capture probe. The resulting product comprises the complex bound to the solid phase via the duplex formed by the oligonucleotide bound to the solid phase and the second binding sequence of the capture probe. The solid phase with bound complex is then separated from unbound materials.

The multimer is then added to the solid phase-analyte-probe complex under hybridization conditions to permit the multimer to hybridize to the available second binding sequences of the amplifier probe of the complex. The resulting solid phase complex is then separated from any unbound multimer by washing. The labeled oligonucleotide is then added under conditions which permit it to hybridize to the complementary oligonucleotide units of the multimer. The resulting solid phase labeled nucleic acid complex is then separated from excess labeled oligonucleotide, by washing to remove unbound labeled oligonucleotide, and read.

The amplification may be multiplied by the use of more than one multimer in the assay. In such instances a first multimer is designed to function as or to bind to the amplifier probe and to a second multimer and the second multimer is designed to bind to the first multimer and to the labeled oligonucleotide. Any number of multimers may be bound in series in this manner to achieve even greater amplification.

The analyte HBV nucleic acids will typically be derived from biological fluids, and may be prepared for the hybridization analysis by a variety of means, e.g., proteinase K/SDS, chaotropic salts, etc. Also, it may be

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of advantage to decrease the average size of the analyte nucleic acids by enzymatic, physical or chemical means, e.g., restriction enzymes, sonication, chemical degradation (e.g., metal ions), etc. The fragments may be as small as 0.1 kb, usually being at least about 0.5 kb and may be 1 kb or higher. The analyte sequence is provided in single-stranded form for analysis. Where the sequence is naturally present in single-stranded form, denaturation will not be required. However, where the sequence is present in double-stranded form, the sequence will be denatured. Denaturation can be carried out by various techniques, such as alkali, generally from about 0.05 to 0.2 M hydroxide, formamide, salts, heat, or combinations thereof.

The first binding sequences of the capture probe and amplifier probe that are complementary to the analyte HBV nucleic acid will each be of at least 15 nucleotides (nt), usually at least 25 nt, and not more than about 5 kb, usually not more than about 1 kb, preferably not more than about 100 nt. They are will typically be approximately 30 nt. They are chosen to bind to different sequences of the consensus sequence for the conserved ds region of the HBV genome.

The second binding sequences of the capture probe and amplifier probe are selected to be complementary, respectively, to the oligonucleotide attached to the solid phase and to an oligonucleotide unit of the multimer and so as to not be encountered by endogenous sequences in the sample/analyte. The second binding sequence may be contiguous to the first binding sequence or be spaced therefrom by an intermediate noncomplementary sequence. The probes may include other noncomplementary sequences if desired. These noncomplementary sequences must not hinder the binding of the binding sequences or cause nonspecific binding to occur.

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The capture probe and amplifier probe may be prepared by oligonucleotide synthesis procedures or by cloning, preferably the former.

The solid phase that is used in the assay may be particulate or be the solid wall surface of any of a variety of containers, e.g., centrifugal tubes, columns, microtiter plate wells, filters, tubing, etc. When particles are used, they will preferably be of a size in the range of about 0.4 to 200 microns, more usually from about 0.8 to 4.0 microns. The particles may be any convenient material, such as latex, or glass. Microtiter plates are a preferred solid surface. The oligonucleotide that is complementary to the second binding sequence of the capture probe may be stably attached to the solid surface through functional groups by known procedures.

It will be appreciated that one can replace the second binding sequence of the capture probe and the oligonucleotide attached to the solid phase with an appropriate ligand-receptor pair that will form a stable bond joining the solid phase to the first binding sequence of the capture probe. Examples of such pairs are biotin/avidin, thyroxine/thyroxine-binding globulin, antigen/antibody, carbohydrate/lectin, and the like.

The labeled oligonucleotide will include a sequence complementary to the second oligonucleotide units of the multimer. The labeled oligonucleotide will include one or more molecules ("labels"), which directly or indirectly provide for a detectable signal. The labels may be bound to individual members of the complementary sequence or may be present as a terminal member or terminal tail having a plurality of labels. Various means for providing labels bound to the sequence have been reported in the literature. See, for example, Leary et al., Proc Natl Acad Sci USA (1983) 80:4045; Renz and Kurz, Nucl Acids Res (1984) 12:3435; Richardson and Gumpert, Nucl Acids Res (1983) 11:6167; Smith et al., Nucl Acids

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Res (1985) 13:2399; Meinkoth and Wahl, Anal Biochem (1984) 138:267. The labels may be bound either covalently or non-covalently to the complementary sequence. Labels which may be employed include radionuclides, fluorescers, chemiluminescers, dyes, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, enzyme subunits, metal ions, and the like. Illustrative specific labels include fluorescein, rhodamine, Texas red, phycoerythrin, umbelliferone, luminol, NADPH, alpha-beta-galactosidase, horseradish peroxidase, etc.

The labeled oligonucleotide can be conveniently prepared by chemical synthesis such as that described in EPA Publication No. 0225807. By providing for a terminal group which has a convenient functionality, various labels may be joined through the functionality. Thus, one can provide for a carboxy, thiol, amine, hydrazine or other functionality to which the various labels may be joined without detrimentally affecting duplex formation with the sequence. As already indicated, one can have a molecule with a plurality of labels joined to the sequence complementary to the labeling sequence. Alternatively, one may have a ligand bound to the labeling sequence and use a labeled receptor for binding to the ligand to provide the labeled analyte complex.

The ratio of capture probe and amplifier probe to anticipated moles of analyte will each be at least stoichiometric and preferably in excess. This ratio is preferably at least about 1.5:1, and more preferably at least 2:1. It will normally be in the range of 2:1 to 10,000:1. Concentrations of each of the probes will generally range from about  $10^{-9}$  to  $10^{-6}$  M, with sample nucleic acid concentrations varying from  $10^{-21}$  to  $10^{-12}$  M. The hybridization steps of the assay will generally take from about 10 minutes to 2 hours, frequently being completed in about 1 hour. Hybridization can be carried

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out at a mildly elevated temperature, generally in the range from about 20°C to 80°C, more usually from about 35°C to 70°C, particularly 65°C.

The hybridization reaction is usually done in an aqueous medium, particularly a buffered aqueous medium, which may include various additives. Additives which may be employed include low concentrations of detergent (0.1 to 1%), salts, e.g., sodium citrate (0.017 to 0.170 M), Ficoll, polyvinylpyrrolidone, carrier nucleic acids, carrier proteins, etc. Nonaqueous solvents may be added to the aqueous medium, such as dimethylformamide, dimethylsulfoxide, alcohols, and formamide. These other solvents will be present in amounts ranging from 2 to 50%.

The stringency of the hybridization medium may be controlled by temperature, salt concentration, solvent system, and the like. Thus, depending upon the length and nature of the sequence of interest, the stringency will be varied.

The procedure used in the separation steps of the assay will vary depending upon the nature of the solid phase. For particles, centrifugation or filtration will provide for separation of the particles, discarding the supernatant or isolating the supernatant. Where the particles are assayed, the particles will be washed thoroughly, usually from one to five times, with an appropriate buffered medium, e.g., PBS containing a detergent such as SDS. When the separation means is a wall or support, the supernatant may be isolated or discarded and the wall washed in the same manner as indicated for the particles.

Depending upon the nature of the label, various techniques can be employed for detecting the presence of the label. For fluorescers, a large number of different fluorometers are available. For chemiluminescers, luminometers or films are available. With enzymes, a fluorescent, chemiluminescent, or colored product can be

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provided and determined fluorometrically, luminometrically, spectrophotometrically or visually. The various labels which have been employed in immunoassays and the techniques applicable to immunoassays can be employed with the subject assays.

Kits for carrying out amplified nucleic acid hybridization assays according to the invention will comprise in packaged combination the following reagents: the multimer; an appropriate labeled oligonucleotide; solid phase that is capable of binding to the analyte; a set of capture probes; and a set of amplifier probes. These reagents will typically be in separate containers in the kit. The kit may also include a denaturation reagent for denaturing the analyte, hybridization buffers, wash solutions, enzyme substrates, negative and positive controls and instructions for carrying out the assay.

The following examples of the invention are offered by way of illustration and not by way of limitation.

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#### Examples

##### 1. Enzymatic Preparation of Linear Multimer

A linear multimer of an 18-mer that is complementary to amplifier probes (see 3.F. below) was prepared according to the scheme depicted in FIG. 1.

Two 18-mers, LLA-1 and LLA-2, were synthesized by an automated phosphoramidite method as described in Warner et al., DNA (1984) 3:401. Purification was carried out according to Sanchez-Pescador and Urdea, DNA (1984) 3:339. Phosphorylation of the 5' end of the LLA-2 was carried out by the chemical phosphorylation procedure described in Example 1 of commonly owned copending EPA Publication No. 0304215, the disclosure of which is incorporated herein by reference. The sequences of LLA-1 and LLA-2 were as follows.



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5' -CGTGTCAGGCATAGGACC

LLA-1

TCCGTATCCTGGGCACAG-p-5'

LLA-2

5 The linear LLA-2 polymer was formed using T4 DNA  
ligase. The products of such ligations are double-  
stranded. Single-stranded polymer may be obtained by gel  
purifying the product under denaturing conditions.

10 One hundred thousand pmole of each sequence were  
added to a 1.5 ml tube and evaporated to dryness under  
vacuum.

The following solution was added to the dried  
sequences:

15 100 ul KBTS buffer\*  
100 ul 10 mM DTT  
100 ul 10 mM ATP  
50 ul H<sub>2</sub>O  
50 ul 1M NaCl  
20 500 ul 30% PEG.

\*10X (50 mM Tris HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 1 mg/ml  
spermidine)

25 The tube was vortexed and then heated to 55°C  
for 30 min. The tube was then cooled to room temperature  
and the following solution was added

30 6.7 ul T4 DNA Ligase (New England Nuclear  
15 U/ul)  
18.8 ul 5X Ligase Dilution Buffer (NEN)  
74.5 ul H<sub>2</sub>O

35 Again the tube was vortexed and then incubated at room  
temperature overnight. The reaction mixture was extracted  
with n-butanol to a volume of 100 ul, 100 ul of 3X stop

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mix (25% glycerol, 0.05% bromphenol blue, 0.5% sodium dodecyl sulfate, 25 mM EDTA) was added and then heated to 100°C for 5 min. to denature the sample. Twenty ul portions of this solution were then added to the wells of a 5 7% denaturing polyacrylamide gel (10 cm x 10 cm x 1.5 mm). The gel was run at 10 V/cm until the bromphenol blue dye was within 0.5 cm of the bottom of the gel. The products formed were made visible by placing the gel on a thin layer chromatography plate containing a UV fluorescing dye 10 covered with Saran wrap and illuminating with a long wave UV lamp. The products absorb the UV irradiation and cast a visible shadow. Bands of increasing length were observed and polymeric products greater than twenty oligomeric units long were cut out. These products were 15 eluted from the gel by soaking in Maxim-Gilbert buffer (0.1 M Tris-HCl, pH 8, 0.5 M NaCl, 5 mM EDTA).

## 2. Chemical Preparation of Linear and Branched Multimers

### 20 A. Preparation of Linear and Branched Multimers

Linear and branched multimers of an 18-mer that are complementary to the amplifier probes (see 3.F. below) were prepared according to the schemes depicted in FIGS. 2A and 2B.

25 As indicated, the schemes use oligonucleotide units having derivatized bases that are cross-linked with phenyldiisothiocyanate (DITC).

The oligonucleotide used to prepare the linear multimer had the sequence

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where X represents the N<sup>4</sup>-(6-aminocaproyl-2-aminoethyl) derivative of cytidine.

35 The oligonucleotide used for the branched multimer had the sequence

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5'-XTGGTCCTATGCCTGACACGTXTGGTCCTATGCCTGACACGTXT-3'

where X is as above.

5           The N<sup>4</sup>-(6-aminocaproyl-2-aminoethyl) derivative of cytidine was prepared as described in EPA Publication No. 0225807. The oligomers were synthesized and purified as described in Example 1.

10           A sample of 0.2 OD260 units of either fragment was dissolved in 0.5 ul of 0.1 M sodium borate, pH 9.3, to which 9.5 ul of DITC in dimethylformamide (2 mg/ml) was added. The solution was vortexed and set at room temperature overnight in the dark. After the addition of 300 ul of n-butanol and mixing, 300 ul of water was added.  
15 The mixture was vortexed and centrifuged to separate the layers. The sample was extracted several times until the aqueous phase was lowered to a volume of approximately 50 ul and then vacuumed to dryness. The polymer was then treated with 10 ul of 1 M glycine, pH 9.5, for 2 hr to  
20 modify any remaining isothiocyanate groups. The mixture was loaded onto a 10 ml Sephadex G-25 column, eluted with water, collected, evaporated to dryness, taken up in 1% SDS and loaded onto a 7% polyacrylamide gel (vertical 2% agarose gels are preferred for preparation runs). The gel  
25 was run at 60 ma and then stained with ethidium bromide. Bands estimated to comprise 10-25 units of the oligomer were cut, electroeluted and precipitated.

#### B. Preparation of Comb-like and Bifurcated Multimers

30           In this section DMT = dimethoxytrityl; T = deoxythymidine; DMF = dimethylformamide; BDMS = t-butyldimethylsilyl; C = deoxycytidine; TLC = thin-layer chromatography; DMAP = N,N-dimethylaminopyridine; THF = tetrahydrofuran; DIPEA = diisopropylethylamine; LEV =  
35 levulinic ester; DCA = dichloroacetic acid; DCC = dicyclohexylcarbodiimide; DCHU = dicyclohexylurea; TEA =

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triethylamine; TMS = trimethylsilyl; Fmoc = 9-fluorenyl-methoxycarbonyl.

5 B.1A. Synthesis of Nucleotide for Forming Comb-Like Branch Points

5-DMT-T-OH (27.3 g, 50 mmole) and imidazole (10 g, 150 mmole) were coevaporated with 200 ml DMF. The residue was dissolved in 250 ml DMF, and BDMS chloride (75 mmol) was added. The reaction mixture was stirred for 18  
10 hr at 20°C. Methanol (50 ml) was added and after 30 min the solvents were removed in vacuo. The oily residue was dissolved in 50 ml ethyl acetate, and the organic phase extracted with 5% aqueous NaHCO<sub>3</sub> (2 x 500 ml) and 80% saturated aqueous NaCl (500 ml) and finally dried over  
15 solid Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed in vacuo to give 35 g (50 mmole) 5'-DMT-3'-BDMS T (100% yield). This material was used without further purification.

Triazole (25.6 g) was suspended in 400 ml of CH<sub>3</sub>CN (at 0°C) and POCl<sub>3</sub> (8 ml) was added with rapid stir-  
20 ring. Then triethylamine (60 ml) was added dropwise over 15 min to the slurry stirred at 0°C for 30 min. 5'-DMT-3'-BDMS T (25 mmole crude) dissolved in 100 ml CH<sub>3</sub>CN was added dropwise to the above stirred slurry at 0°C. The ice-water bath was removed and stirring continued at 20°C  
25 for one hour. The reaction mixture was diluted with 800 ml ethyl acetate, and the organic phase was extracted with 5% NaHCO<sub>3</sub> (2 x 500 ml) and 80% saturated aqueous NaCl (500 ml). After drying the organic phase over solid Na<sub>2</sub>SO<sub>4</sub> solvents were removed in vacuo. The resulting residue was  
30 coevaporated with toluene (400 ml) and CH<sub>3</sub>CN (400 ml) to give 5'-DMT-3'-BDMS-5-methyl-4-triazoyl  $\beta$ -D-2-deoxyribofuranosyl-2(1H)-pyrimidinone as a white foam in quantitative yield. This material was used without further purification.

35 To a solution of 6-aminohexanol (11.7 g, 100 mmole) in 400 ml CH<sub>3</sub>CN was added dropwise 5'-DMT-3'-BDMS-

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5-methyl-4-triazoyl beta-D-2-deoxyribofuranosyl-2(1H)-pyrimidinone (8.7 g, 12 mmole) dissolved in 100 ml CH<sub>3</sub>CN and the reaction mixture stirred at 20°C. The progress of the reaction was monitored by TLC (every 30 min) and when the starting material had completely disappeared (usually in 1-2 hours), the reaction mixture was diluted with 500 ml ethyl acetate, which was extracted with 5% aqueous NaHCO<sub>3</sub> and 80% saturated aqueous NaCl as described above. After drying of the organic phase over Na<sub>2</sub>SO<sub>4</sub>, the solvent was removed in vacuo to give 7.0 g (9.2 mmole) of product 5'-DMT-3'-BDMS-5-methyl-N<sup>4</sup>-6-hydroxyhexyl deoxycytidine (yield 77%). This material was used without further purification.

To a solution of 5'-DMT-3'-BDMS-5-methyl-N<sup>4</sup>-6-hydroxyhexyl deoxycytidine (7 g, 9.2 mmole) in 100 ml THF was added (CH<sub>3</sub>COCH<sub>2</sub>CH<sub>2</sub>CO)<sub>2</sub>O (50 mmole) dissolved in 100 ml THF and then 10 ml 6.5% DMAP in 2,6-lutidine/THF. The reaction mixture was left stirring for 30 min. The analysis showed that starting material had been completely consumed. The reaction mixture was diluted with 700 ml ethyl acetate which was diluted with 700 ml ethyl acetate which was extracted with 5% aqueous NaHCO<sub>3</sub> (3 x 500 ml) and 80% saturated aqueous NaCl (500 ml) as described above. After drying over solid Na<sub>2</sub>SO<sub>4</sub>, the solvent was removed and the residue coevaporated with toluene (200 ml) and CH<sub>3</sub>CN (200 ml) to give 12.3 g of crude product.

This crude product was dissolved in 100 ml THF, and 10 ml of a 1.1 M solution of tetrabutylammonium fluoride in THF was added. The progress of the reaction was monitored by TLC; it is usually over in 30 min but may take longer. When starting material had been consumed, the reaction mixture was diluted with 700 ml ethyl acetate, which was extracted with NaHCO<sub>3</sub> and NaCl solutions, as above. Removal of the solvent afforded 8.5 g crude product 5'-DMT-5-methyl-N<sup>4</sup>(O-levulinyl-6-oxyhexyl)-2'-deoxycytidine. This material was subjected to silica

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gel chromatography. The purified product was isolated by elution with 4% methanol in  $\text{CH}_2\text{Cl}_2$  to give 5.0 g of a slightly brownish foam (6.7 mmole; 73% yield).

Silica-purified 5'-DMT-5-methyl- $\text{N}^4$ (O-levulinyl-6-oxyhexyl)-2'-deoxycytidine (7.7 mmole) was coevaporated twice with  $\text{CH}_3\text{CN}$ . The resulting dry powder was dissolved in 70 ml  $\text{CH}_2\text{Cl}_2$  containing 4.9 ml DIPEA in a flask under argon. After cooling to  $0^\circ\text{C}$ , 1.65 ml (8.5 mmole) N,N-diisopropylaminomethoxy chlorophosphine was added with a syringe and the mixture stirred at  $0^\circ$  for 30 min. After dilution with 400 ml ethyl acetate, the organic phase was washed 4 times with 400 ml 80% saturated aqueous NaCl, then dried over solid  $\text{Na}_2\text{SO}_4$  and filtered. The solvent was removed in vacuo and the resulting residue coevaporated twice with toluene to give an oil. This oil was dissolved in 30 ml toluene and added dropwise into 400 ml cold hexane ( $\sim -20^\circ\text{C}$ ). The precipitate was quickly collected by filtration and dried in vacuo for 18 hr to give 5.45 g of phosphoramidite (6.0 mmole; 78% yield).

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B.1B. Synthesis of Alternative, Preferred Nucleotide for Forming Comb-Like Branch Points

To a solution of 5'-DMT-3-BDMS-5-methyl- $\text{N}^4$ -6-hydroxyhexyl deoxycytidine (34 g, 50 mmole) prepared as described above in 200 ml  $\text{CH}_2\text{Cl}_2$  was added 1.5 g N,N-dimethylaminopyridine and 25 ml triethylamine. To this solution at  $0^\circ\text{C}$  was added dropwise DMT-Cl (75 mmole, 25.5 g) dissolved in  $\text{CH}_2\text{Cl}_2$  (100 ml). The reaction mixture was left stirring for 1 hour. The analysis showed that starting material had been completely consumed. Then 50 ml of methanol was added. After 30 min the reaction mixture was diluted with 800 ml ethyl acetate which was extracted with 5%  $\text{NaHCO}_3$  (2 x 500 ml) and 80% saturated aqueous NaCl (500 ml) described above. After drying over solid  $\text{Na}_2\text{SO}_4$  the solvent was removed in vacuo and the

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residue coevaporated with toluene (200 ml) and  $\text{CH}_3\text{CH}$  (200 ml).

This crude product was dissolved in 200 ml THF, and 200 ml of a 1.1 M solution of tetrabutylammonium fluoride in THF was added. The progress of the reaction was monitored by TLC; it is usually over in 30 min but may take longer. When starting material had been consumed, the reaction mixture was diluted with 700 ml ethyl acetate, which was extracted with  $\text{NaHCO}_3$  and  $\text{NaCl}$  solutions, as above. Removal of the solvent afforded 36 g crude product, 5'-DMT-5-methyl- $\text{N}^4$ (O-DMT-6-oxyhexyl)deoxycytidine. This material was subjected to silica gel chromatography. The purified product was isolated by elution with 2-4% methanol in  $\text{CH}_2\text{Cl}_2$  to give 32.7 g of pure product (34 mmole; yield based on 5'-DMT-T-OH: 69%).

Silica-purified 5'-DMT-5-methyl- $\text{N}^4$ (O-DMT-6-oxyhexyl)-2'-deoxycytidine (34 mmole) was coevaporated twice with  $\text{CH}_3\text{CN}$ . The resulting dry powder was dissolved in 100 ml  $\text{CH}_2\text{Cl}_2$  containing 7.5 ml DIPEA in a flask under argon. After cooling to  $0^\circ\text{C}$ , 7.37 ml (38 mmole)  $\text{N,N}$ -diisopropylaminomethoxy chlorophosphine was added with a syringe and the mixture stirred at  $0^\circ$  for 30 min. After dilution with 800 ml ethyl acetate, the organic phase was washed 4 times with 800 ml 80% saturated aqueous  $\text{NaCl}$ , then dried over solid  $\text{Na}_2\text{SO}_4$  and filtered. The solvent was removed in vacuo and the resulting residue coevaporated twice with toluene to give an oil. This oil was dissolved in 80 ml toluene and added dropwise into 700 ml cold hexane ( $\sim -20^\circ\text{C}$ ). The precipitate was quickly collected by filtration and dried in vacuo for 18 hr to give 31.8 g of phosphoramidite (28.7 mmole; 84% yield).

5'-DMT-T-OH (16.4, 30 mmole) was dissolved in dry 200 ml  $\text{CH}_3\text{CN}$  and 1-(TMS)imidazole (14.6 ml, 100 mmole) was added. After 60 min the solvents were removed in vacuo. The oily residue was dissolved in 700 ml ethyl acetate, and the organic phase extracted with 5% aqueous

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NaHCO<sub>3</sub> (2 x 500 ml) and 80% saturated aqueous NaCl (500 ml) and finally dried over solid Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed in vacuo to give 30 mmole 5'-DMT-3'-TMS-T (100% yield). This material was used without further purification.

Triazole (37.8 g) was suspended in 450 ml of CH<sub>3</sub>CN (at 0°C) and POCl<sub>3</sub> (12 ml) was added with rapid stirring. Then triethylamine (90 ml) was added dropwise over 15 min to the slurry stirred at 0°C for 30 min. 5'-DMT-3'-TMS-T (30 mmole crude) dissolved in 100 ml CH<sub>3</sub>CN was added dropwise to the above stirred slurry at 0°C. The ice-water bath was removed and stirring continued at 20°C for one hour. The reaction mixture was diluted with 800 ml ethyl acetate, and the organic phase was extracted with 5% NaHCO<sub>3</sub> (2 x 500 ml) and 80% saturated aqueous NaCl (500 ml). After drying the organic phase over solid Na<sub>2</sub>SO<sub>4</sub> solvents were removed in vacuo. The resulting residue was coevaporated with toluene (400 ml) and CH<sub>3</sub>CN (400 ml) to give 5'-DMT-3'-TMS-5-methyl-4-triazoyl β-D-2-deoxyribofuranosyl-2(1H)-pyrimidinone as a white foam in quantitative yield. This material was used without further purification.

To a solution of 6-aminohexanol (23 g, 200 mmole) in 400 ml CH<sub>3</sub>CN was added dropwise 5'-DMT-3'-TMS-5-methyl-4-triazoyl beta-D-2-deoxyribofuranosyl-2(1H)-pyrimidinone (20 g, 30 mmole) dissolved in 100 ml CH<sub>3</sub>CN and the reaction mixture stirred at 20°C. The progress of the reaction was monitored by TLC (every 30 min) and when the starting material had completely disappeared (usually in 1-2 hours), the reaction mixture was diluted with 800 ml ethyl acetate, which was extracted with 5% aqueous NaHCO<sub>3</sub> and 80% saturated aqueous NaCl as described above. After drying of the organic phase over Na<sub>2</sub>SO<sub>4</sub>, the solvent was removed in vacuo to give 20.3 g (~30 mmole) of product 5'-DMT-3'-TMS-5-methyl-N<sup>4</sup>-6-hydroxyhexyl deoxycytidine. This material was used without further purification.



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To a solution of 5'-DMT-3'-TMS-5-methyl-N<sup>4</sup>(6-hydroxyhexyl)deoxycytidine in 250 ml methanol was added 25 ml concentrated aqueous NH<sub>4</sub>OH and the reaction mixture left stirring in a closed round-bottom flask to 1 hour.

- 5 The solvent was then removed in vacuo and coevaporated with 1 x 200 ml ethanol, 1 x 100 ml toluene and 1 x 100 ml CH<sub>3</sub>CN to give 5'-DMT-5-methyl-N<sup>4</sup>(6-hydroxyhexyl)deoxycytidine in quantitative yield. This material was used without further purification. This material was dissolved
- 10 in 200 ml CH<sub>2</sub>Cl<sub>2</sub> and 4 ml of pyridine was added followed by dropwise addition of Fmoc-Cl (7.8 g, 30 mmole) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (50 ml). The reaction mixture was left stirring for 30 min. The analysis showed that starting material had been completely consumed. The reaction
- 15 mixture was diluted with 500 ml ethyl acetate which was extracted with 5% aqueous NaHCO<sub>3</sub> (3 x 500 ml) and 80% saturated aqueous NaCl (500 ml) as described above. After drying over solid Na<sub>2</sub>SO<sub>4</sub>, the solvent was removed and the residue coevaporated with toluene (200 ml) and CH<sub>3</sub>CN (200
- 20 ml) to give 23.7 g of crude product. This crude product was subjected to silica gel chromatography. The purified product eluted with about 4% methanol in CH<sub>2</sub>Cl to give 13.3 g (15.3 mmole) of pure 5'-DMT-5-methyl-N<sup>4</sup>(O-Fmoc-6-oxyhexyl)deoxycytidine (50% yield based on 5'-DMT-TOH).
- 25 Silica-purified 5'-DMT-5-methyl-N<sup>4</sup>(O-Fmoc-6-oxyhexyl)-2'-deoxycytidine (15.3 mmole) was coevaporated twice with CH<sub>3</sub>CN. The resulting dry powder was dissolved in 60 ml CH<sub>2</sub>Cl<sub>2</sub> containing 4.1 ml DIPEA in a flask under argon. After cooling to 0°C, 3.19 ml (16.5 mmole) N,N-diisopropylaminomethoxy chlorophosphine was added with a
- 30 syringe and the mixture stirred at 0° for 30 min. After dilution with 400 ml ethyl acetate, the organic phase was washed 4 times with 400 ml 80% saturated aqueous NaCl, then dried over solid Na<sub>2</sub>SO<sub>4</sub> and filtered. The solvent
- 35 was removed in vacuo and the resulting residue coevaporated twice with toluene to give an oil. This oil

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was dissolved in 50 ml toluene and added dropwise into 400 ml cold hexane ( $\sim -20^{\circ}\text{C}$ ). The precipitate was quickly collected by filtration and dried in vacuo for 18 hr to give 12.15 g of phosphoramidite (11.8 mmole; 77% yield).

- 5 Removal of O-FMOC group during solid phase synthesis: t-butylamine/pyridine (1:10 v/v) for 1 hour at  $20^{\circ}$ . Removal of O-levulinyl group: 0.5 M hydrazine hydrate in pyridine/glacial acetic acid (4:1 v/v) 15 minutes at  $20^{\circ}\text{C}$ .

10 B.2. Synthesis of a Multifunctional Phosphoramidite for Forming Bifurcated Branch Points

- Glycerol (10 mmole) was dried by coevaporation with pyridine. The resulting oil was dissolved in 50 ml pyridine and DMT-Cl (6.8 g, 20 mmole) was added, and the  
15 reaction mixture was stirred at  $20^{\circ}\text{C}$  for 18 hr. After addition of methanol (10 ml), the solvent was removed on a rotary evaporator. The resulting oil was dissolved in 250 ml ethyl acetate and the organic phase was washed with 5% aqueous  $\text{NaHCO}_3$  (2 x 250 ml), 80% saturated aqueous NaCl (1  
20 x 250 ml) and then dried over solid  $\text{Na}_2\text{SO}_4$ . The solvent was removed in vacuo and the residue coevaporated with 100 ml toluene and 100 ml  $\text{CH}_3\text{CN}$ . The product was isolated by silica gel chromatography to give 2.5 g (3.6 mmole) 0,0-bis DMT glycerol (35% yield). Product elutes with 0-1%  
25 MeOH.

- The bis-DMT glycerol (2.3 mmole) was dissolved in  $\text{CH}_2\text{Cl}_2$  (10 ml) containing DIPEA (0.8 ml) under argon, and N,N-diisopropylamino-2-cyanoethoxy-chlorophosphine (1 ml) was added by syringe. After 30 minutes the mixture  
30 was diluted with ethyl acetate (200 ml) and the organic phase washed with 200 ml 80% saturated aqueous NaCl. After drying over solid  $\text{Na}_2\text{SO}_4$ , the solvent was removed in vacuo, and the residue coevaporated with 100 ml toluene and 100 ml dry  $\text{CH}_3\text{CN}$  to give 2.15 g (2.3 mmole; 100%  
35 yield) of bis-DMT glycerol phosphoramidite. The product was dispensed with dry  $\text{CH}_3\text{CN}$  into small septum-capped vi-

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als and after removal of the solvent stored at  $-20^{\circ}\text{C}$  under argon.

B.3. Synthesis of Periodate-Cleavable Linker  
5 Phosphoramidite

O,O-Dibenzoyl tartaric acid monohydrate (18.8g, 50 mmole) was dissolved in 250 ml  $\text{CH}_3\text{CN}$  and the solvent was removed in vacuo. This process was repeated. The resulting oil was dissolved in 250 ml THF and DCC (10.6g, 10 50 mmole) dissolved in 50 ml THF was added. A precipitate started forming in a few minutes. After stirring for 18 hr at  $20^{\circ}\text{C}$  the reaction mixture was filtered, and the precipitate washed with THF. The precipitate was dried in high vacuum to give 10.8g (50 mmole) DCHU. To the 15 combined filtrate was added 2-(N-methyl)aminoethanol (4.0 ml, 50 mmole) and the reaction mixture was stirred for 1 hr at  $20^{\circ}\text{C}$ . DCC (10.6g, 50 mmole) in 50 ml THF was then added. A small precipitate formed. After about 1 hr, 2-(N-methyl)aminoethanol (4.0 ml, 50 mmole) was added and 20 the reaction mixture stirred for 18 hours at  $20^{\circ}\text{C}$ .

The formed precipitate was filtered off and washed with THF. The dried precipitate of DCHU weighed 10.8g. The combined filtrate was evaporated to an oil. Chromatography on silica afforded 8g (17 mmole) of O,O-dibenzoyl tartaric di(N-methyl-2-hydroxyethyl)amide (this 25 product elutes with 6%  $\text{MeOH}/\text{CH}_2\text{Cl}_2$ ).

To the amide product (8.6 mmole) in 50 ml  $\text{CH}_2\text{Cl}_2$  containing DMAP (0.11 g) and TEA (2.4 ml) was added dropwise, DMT-Cl (8.6 mmole) dissolved in 50 ml  $\text{CH}_2\text{Cl}_2$ . 30 After addition of DMT-Cl the reaction mixture was stirred for 1 hr at  $20^{\circ}\text{C}$ , then the solvent was removed by evaporation. The residue was dissolved in 600 ml ethyl acetate and the organic phase washed with 400 ml 5%  $\text{NaHCO}_3$  and 400 ml 80% saturated aqueous NaCl. The organic phase was 35 dried over solid  $\text{Na}_2\text{SO}_4$ . After 30 min the  $\text{Na}_2\text{SO}_4$  was filtered off, and the supernatant was concentrated to an

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oil, then coevaporated with toluene and  $\text{CH}_3\text{CN}$ . The crude material was subjected to silica gel chromatography using  $n$ -butanol/ $\text{CH}_2\text{Cl}_2$  for elution. The pure mono-DMT product eluted with 2-3%  $n$ -butanol/ $\text{CH}_2\text{Cl}_2$  to give 1.53 g (2 mmole) of  $O,O$ -dibenzoyl tartaric 2-( $O$ -dimethoxytrityl)hydroxyethyl- $N,N$ -dimethyl,  $N$ -methyl-2-hydroxyethylidiamide.

This material was dissolved in 20 ml  $\text{CH}_2\text{Cl}_2$  containing DIPEA (3 mmole). After cooling to  $10^\circ\text{C}$ , 2.2 mmole methoxy- $N,N$ -diisopropylaminochlorophosphine was added under argon. After 15 min, ethyl acetate was added, and the organic phase washed with 80% saturated aqueous  $\text{NaCl}$ , dried over solid  $\text{Na}_2\text{SO}_4$  and evaporated to dryness. After coevaporation with toluene and dry  $\text{CH}_3\text{CN}$ , the phosphoramidite residue was dissolved in 10 ml dry  $\text{CH}_3\text{CN}$ . This solution was aliquoted into 19 dry Weaton vials and the solvent removed in vacuo. The vials were closed with septum screw caps and stored at  $-20^\circ\text{C}$ .

This phosphoramidite may be coupled to oligonucleotides using standard DNA synthesis techniques and equipment. After deprotection the resulting linker-containing DNA may be cleaved at the 1,2-diol site as described above.

#### B.4. Synthesis of a Four-Site Bifurcated Amplification Multimer

For synthesis of branched fragments, a 2000 A control pore glass (CPG) support (30 mg of 7.3  $\mu\text{mole}$  of nucleoside/g) was used. The CPG support was synthesized as described in EPA Publication No. 0304215. An automated methyl phosphoramidite coupling procedure was employed for DNA synthesis as above, except that during the addition of the branched oligonucleotide segments 2.5% (v/v) DCA in toluene (instead of  $\text{CH}_2\text{Cl}_2$ ) was used for detritylation.

Fragment LLA-2 (GACACGGGTCCTATGCCT; see above) was synthesized on the CPG, then removed from the

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automated synthesizer and placed into a sintered glass funnel. Manual phosphoramidite couplings were then performed as described (Urdea, M.S., Methods in Enzymol (1987) 146:22-41). A 100 umole aliquot of the DMT<sub>2</sub>-glycerol phosphoramidite (2-cyanoethyl form) and 250 moles of tetrazole were added to the 5'-deprotected fragment and incubated for 15 min. This process was then repeated. Two T methyl phosphoramidites were then added manually and an additional glycerol phosphate was added. The CPG was then placed back on the automated synthesizer and the fragment GATGTGGTTGTCGTACTTTT was synthesized off of each branch. The standard deprotection and purification as above was then employed.

15 B.5. Synthesis of a Four- and Five-Site Comb Amplification Multimer

Using the methods described above, the fragment TTOTTOTTOTTGACACGGGTCCTATGCCT (O = 5'-DMT-N<sup>4</sup>-[LevO(CH<sub>2</sub>)<sub>6</sub>]-5-Me cytidine methyl phosphoramidite) was synthesized. The fragment was then removed from the machine and treated for 1 hr at room temperature with 0.5 M hydrazine monohydrate in 8:2 (v/v) pyridine/concentrated acetic acid. The CPG was then washed with pyridine, then 1:1:2 (v/v/v) H<sub>2</sub>O/lutidine/THF. The support was then placed back on the automated synthesizer and the fragment was synthesized off of each O in the above formula. The standard deprotection and purification as above was then employed. Note that one copy of the labeled probe (LLA-2) binding fragment is at the 5'-end of the multimer and three copies of the fragment are at the O residues.

A five-site multimer was made in a similar manner.

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3. Sandwich Hybridization Assay for Hepatitis B Virus (HBV) DNA Using Multimer

FIG. 4 schematically depicts the assay procedure.

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A. Standard Analyte HBV DNA

The plasmid pHE63 composed of the entire 3.2 kb HBV genome cloned into the EcoRI site of plasmid pBR325 linearized with EcoRI and diluted into normal human serum was used as standard analyte. The analyte is designated 11 in FIG. 4.

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B. Solid Phase Oligonucleotide Complex (C in FIG. 4)

A 21 base oligomer, 5'-XCACCACTTTCTCCAAAGAAG-3', where X is as defined above, was synthesized as described in Example 1 and biotinylated using N-hydroxysuccinimide biotin in 0.1 M sodium phosphate, pH 7.5. A 5 ul aliquot of this biotinylated fragment (800 pmoles) was added to a 1.5 ml Eppendorf tube containing 500 ul of 0.25% (w/v) 2.8 micron avidin polystyrene beads in 1X PBS. After a 1 h incubation at 37°C, the beads were washed 3 times with 500 ul of 0.1% SDS, 4X SSC by centrifugation then resuspended and stored in the same solution until used.

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C. Labeled Oligomer (E in FIG. 4)

An 18 base oligomer, 5'-XGGTCCTAGCCTGACAGC-3', where X is defined as above, was synthesized, modified with DITC in 95:5 (v/v) dimethylformamide:0.1 M sodium borate, pH 9.3, extracted with n-butanol, and combined with horseradish peroxidase (HRP).

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D. Capture Probes (A in FIG. 4)

A set of 12 single-stranded oligomers each having a varying 30 base long portion complementary to a specific sequence of the HBV genome and a constant 20 base

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long 3'-portion complementary to the oligonucleotide bound to the solid phase was synthesized by the procedures described in Example 1.

5                   E. Amplifier Probes (B in FIG. 4)

A set of 36 single-stranded oligomers each consisting of a varying 30 base long portion complementary to a specific sequence of the HBV genome and a constant 20 base long 3'-portion complementary to the multimer (D in  
10 FIG. 4) were synthesized by the procedures described in Example 1.

Both the capture and amplifier probes were designed for the constant ds region of the HBV virus.

15                   F. Bead Assay Procedure

10 ul samples of analyte were placed in 1.5 ml Eppendorf tubes and treated with 12.5  $\mu$ l of proteinase K/SDS (Gene (1987) 61:254) at 37°C for 30 min. To each sample, 5  $\mu$ l of 1 M NaOH containing 50 fmoles each of the  
20 48 HBV oligonucleotide probes (12 capture probes and 36 amplifier probes) were added and the tubes were heated to 100°C for 10 min. The samples were set on ice for 5 min, microfuged for 10 sec and neutralized with 0.38 M acetic acid, 12.3X SSC (final 4X SSC). Annealing of the probes  
25 to the analyte was conducted at 55°C for 1 h. Subsequently, 25  $\mu$ l of the capture beads were added and the solution was left at 55°C for an additional 15 min. Two washes were performed by adding 500  $\mu$ l of wash solution (0.1% SDS, 4X SSC), vortexing, centrifuging for 1 min  
30 and decanting. To each tube, 20  $\mu$ l containing 50 fmoles of multimer (D in FIG. 4) in HM buffer (0.1% SDS, 4X SSC, 1 mg/ml sonicated salmon sperm DNA, 1 mg/ml poly-A, 10 mg/ml BSA) was added. After vortexing, the tubes were left at 55°C for 15 min and washed twice as above. Labeling  
35 was conducted with 20  $\mu$ l containing 250 fmoles of probe Type E in HM for 1 h at 37°C. After three washes as

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above, the beads were thoroughly drained by inversion onto Kimwipes, treated with the appropriate substrate and measured as described below. The total time required for the analysis from addition of the proteinase K/SDS solution to readout was 3 h 50 min in the chemiluminescent format.

The enhanced chemiluminescence method for HRP detection reported by Matthews et al., Anal Biochem (1985) 151:205-209, was employed. Beads were taken up in 15 ul of chemiluminescent substrate solution (luminol with p-hydroxycinnamic acid) and then transferred to 8 x 50 mm Evergreen polypropylene tubes containing 5 ul of 4 mM H<sub>2</sub>O<sub>2</sub>. After 30 sec, tubes were read on a Turner TD-20e luminometer (delay, 10 sec; integration, 20 sec; smoothing, 20 sec). Output was given as the full integral of the light produced during the reaction.

To each tube, a 100 ul aliquot of a fresh o-phenylenediamine solution (OPD; in tablet form from Sigma Chemicals; 50 mg dissolved in 5 ml of 50 mM sodium citrate, pH 5.1, containing 3 ul of 30% H<sub>2</sub>O<sub>2</sub>) was added. After 20 min at 37°C, 50 ul of 4 N H<sub>2</sub>SO<sub>4</sub> was added to quench the reaction. The beads were then pelleted by centrifugation and the supernatant was transferred to a microtiter dish well. The dish was then read on a Biotek EL310 plate reader set at 490 nm. Longer incubations did not improve the signal to noise ratios.

#### G. Microtiter Dish Assay Procedure

A microtiter dish assay procedure was employed. Microtiter dishes were prepared as follows. Two types of microtiter dish wells were prepared: (1) N wells for sample work-up and negative controls, and (2) S wells for capture of the probe-analyte complex from samples and positive controls.

N wells were produced as follows: 300 ul of HM buffer was added to Immulon II Remov-a-wells (Dynatech



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Inc.). The well strips were covered and left standing at room temperature for 1 hour. The HM buffer was removed by aspiration and the wells were washed 3 times with 400 ul of 1X PBS. The strips were covered with plastic wrap and  
5 stored at 4°C until used. Alternatively, wells were coated with poly-phenylalanyl-lysine then with HM buffer, as below.

S wells were prepared from the Immulon II strips as follows. To each well, 200 ul of a 200 ug/ml solution  
10 of poly-phenylalanyl-lysine (Sigma Chemical Inc.) in water. The covered strips were left at room temperature for 30 min to 2 hr, then washed as above. A 10 OD<sub>260</sub> sample of the oligonucleotide of 3B above in 60 ul of 1X PBS was treated with 140 ul of DMF containing 10 mg of  
15 ethylene glycolbis(succinimidylsuccinate) (Pierce Chemicals Inc.). The mixture was vortexed and incubated in the dark at room temperature. After 15 min, the solution was passed over a Sephadex G-25 column (PD-10 from Pharmacia), previously equilibrated with 30 ml of 1X PBS.  
20 The void volume of the column was diluted to a final volume of 35 ml with 1X PBS. To each well, a 50 ul aliquot of the capture probe solution was added. After covering with plastic wrap, the wells were incubated at room temperature in the dark for 30 min to overnight. The  
25 wells were washed with 1X PBS, then coated with H buffer, washed, and stored as above.

Label oligonucleotides were derivatized with alkaline phosphatase (AP) as follows. Calf intestinal AP (3 mg in buffer; immunoassay grade, Boehringer-Mannheim)  
30 was placed in a Centricon 30 Microconcentrator. Approximately 2 ml of 0.1 M sodium borate, pH 9.5, was then added and the device was spun at 3500 rpm until a final volume of 40 ul was obtained. The alkylamino oligonucleotide (Section 3C) was then activated with DITC,  
35 extracted with butanol, and combined with the protein as described for the HRP probe. PAGE, elution (with 0.1 M

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Tris, pH 7.5, 0.1 M NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, and concentration as described for the HRP conjugates were employed. The final product was stored at 4°C.

For duplicate analyses, 20 ul of each sample was placed into 2 N wells, then treated with 25 ul of proteinase K/SDS solution. The wells were covered with a Linbro-Titertek microtiter plate sealer, gently agitated, and incubated at 65°C for 30 min in a water bath. The capture and amplifier probe sets in 1 M NaOH were added in 10 ul to each well. After sealing, the samples were incubated for 10-30 min at 65°C to 72°C as above. The solutions were neutralized with 26 ul 0.38 M acetic acid (or 0.76 M 3-[N-Morpholino]propane sulfonic acid (MOPS), free acid), 12.3X SSC, then incubated for an additional 15-30 min covered at 65°C. From each N well, 40 ul of sample was transferred to a new S well containing the solid supported capture probe. The wells were sealed and set at 65°C for 1-2 hours. Each well was then washed 2 times by aspiration with 0.1% SDS, 0.1X SSC. A solution of amplification multimer (for N. gonorrhoeae, penicillin-resistant N. gonorrhoeae, tetracycline resistant N. gonorrhoeae and Chlamydia tests; a five-site comb structure (section 2.B.5) was employed) in HM buffer was then added and the samples set sealed in a 55°C water bath for 15 min to 1 hour. After washing as above, 20 ul of the enzyme-labeled probe in HM buffer was added. Incubation for the HRP probe was carried out for 15 min at 42°C, while the alkaline phosphatase probe was used at 55°C for 15 min. After washing as above, the appropriate detection reagents were added.

For HRP, the enhanced luminol reagent (see 3F) was used as above.

For AP detection, an enzyme-triggered dioxetane (Schaap et al., Tet Lett (1987) 28:1159-1162 and EPA Publication No. 0254051), obtained from Lumigen Inc., was employed. The detection procedure was as follows. For

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the labeling step 20 ul MM buffer with the AP probe was added to each well and the wells were incubated at 55°C for 15 min. The supernatant was removed and the wells were washed 2X with 380 ul of 0.1X SSC-0.1% SDS. The wells were then washed 2X with 380 ul of 0.1X SSC to remove any remaining SDS. 20 ul of  $3.3 \times 10^{-4}$  M dioxetane in CTAB buffer was added to each well. The wells were tapped lightly so that the reagent would fall to the bottom and gently swirled to distribute the reagent evenly over the bottom. The wells were covered with the microtiter plate sealer and incubated in a 37°C oven for one hour. The wells are then read with a luminometer.

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### Results

#### A. Tests on Standard Analyte

The above described assay was carried out on samples containing known amounts of the standard analyte HBV DNA using the multimers of Examples 1-2A. For comparison purposes a three piece assay (bound analyte; oligonucleotide complementary to both analyte and labeled probe; and HRP-labeled probe) was carried out. The comparison assay was assigned a gain value of 1. Gain is the ratio of signal obtained in an amplified assay to signal obtained in an unamplified three piece assay.

The results of these assays are reported in the table below. S/N = signal-to-background ratio.

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TABLE 1

AMPLIFIER	ASSAY TYPE	GAIN	SIGNAL	BACKGROUND	S/N
Enzymatically Ligated Multimer (Ex. 1)	HBV Serum Assay	29	56.2 ± 24.4 @ 10 amole	12.9 ± 4.73	4.4
Chemically Cross- linked Linear Multimer (Ex. 2)	HBV Serum Assay	25	617 ± 116 @ 50 amole	432 ± 53	1.4
Chemically Cross- linked Branched Multimer (Ex. 2)	HBV Serum Assay	102	168 ± 31 @ 10 amole	17 ± 2	9.9
HRP Probe Without Amplifier	3 Piece Assay	1	32.9 ± 5.7 @ 100 amole	18.2 ± 1.5	1.8

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### B. Tests on Authentic HBV DNA Samples

Authentic HBV DNA samples were identified as follows.

A dot blot screening was conducted for the presence of HBV DNA in 49 HBV surface antigen positive samples using the protein-DNA complex extraction technique of Zyzik et al., Eur J Chem Microbiol (1986) 5:330-335. FIG. 5 presents the analysis of the six DNA positive sera found in the set of 49 using  $^{32}\text{P}$  nick translated pHE63 as a probe. Each sample was blotted and probed in duplicate directly ( $10^0$ ), diluted 1:10 ( $10^1$ ) and 1:100 ( $10^2$ ) in pooled normal human sera. Samples of pHE63 were blotted in duplicate in pooled normal sera or in buffer (10X SSC). Five separate blotting experiments of these sera with dilutions and plasmid standards were performed to establish the ranges calculated. These samples were used in the evaluation of the sandwich hybridization assay of the invention.

FIG. 6 presents the results obtained for the chemiluminescent readout format of the bead capture assay method with the HBV DNA positive samples described above. Analysis of a 4 pg sample of pHE63 is also shown. Two shaded values are given for each sample. The shaded bar represents the absolute signal (S) obtained for each sample and is expressed as the mean for two sets of triplicate samples (6 total samples). The open bar indicates the same number of control samples run on the same sera using beads that do not contain the Type C capture probe. This control, which is not possible in a blotting assay format, was used to determine nonspecific binding or noise (N) for each sample matrix that might lead to false positive signals. The specific signal for each sample can be expressed as the ratio of S and N as defined. An S/N ratio of 1 indicated no specific binding. Minimal S/N ratios indicating a positive sample are discussed below.

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S/N ratios ranged from 3.9 to 49 for sera containing between 0.2 and 8 pg of HBV DNA. Assays conducted on two and four fold dilutions of sera 4825 and 3657 into pooled normal human sera resulted in a near  
5 linear decrease in the absolute signals for each sample, substantiating the presumed specific nature of the method. Excellent run to run reproducibility was found for all samples with different lots of beads and reagents. Although samples were read only 30 sec after addition of  
10 the chemiluminescent substrate solution, equivalent results were obtained for up to 45 min. Also, longer signal integrations did not improve S/N ratios. The sera employed ranged from cloudy to clear in appearance, were stored frozen and freeze-thawed many times. No attempt  
15 was made to clarify the samples prior to hybridization analysis. Increased solution hybridization or bead capture times (up to 18 h) did not significantly increase the S/N ratios.

In FIG. 7 the analysis of subpicogram HBV DNA  
20 samples are compared to known negative sera and large quantities of heterologous nucleic acids in pooled sera. Although the S value for the lowest positive serum employed (upper panel; 1 to 10 dilution of serum 3657) is slightly higher than the highest S for a negative serum  
25 (0092) or non-HBV DNA (HIV), no clear cutoff to differentiate between true and false positives below the pg level was possible based on absolute S alone. However, if the S/N was compared for these same samples (lower panel), a cutoff of S/N = 2.5 permitted a clear distinction to be  
30 made. No negative sample had a value higher than 1.7, yet the lowest positive at 0.2 pg had an S/N = 4.3. This clearly demonstrates the value of using a nonspecific binding control for each sample.

In FIG. 8, a comparison is made between an assay  
35 conducted on HBV DNA positive and negative sera using both the luminol-p-hydroxycinnamic acid (given as relative

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luminescence, RL) and o-phenylenediamine (OPD) detection. Although the methods were comparable in sensitivity, the chemiluminescent method was preferred for several reasons. Firstly, using the bead format, the best colorimetric results were obtained by conducting the OPD reactions in Eppendorf tubes and transferring the solution to a microtiter dish for reading on an ELISA reader since the scattering from beads proved to be a significant source of background. In contrast, beads did not interfere with the chemiluminescent readout on the luminometer. Secondly, the chemiluminescent method was considerably faster. As opposed to waiting 30 min after addition of OPD before the detection, the chemiluminescent reactions could be read rapidly 30 sec after addition of the substrate. Lastly, there was a 1.5- to 2-fold increase in the S/N using RL versus OPD.

#### 4. HBV Subtype Non-specific Sandwich Hybridization Assays

Extensive comparison of the assay designed for adw HBV subtype described in part B of the results section of Example 3 above against dot blots using a <sup>32</sup>P labeled strain adw HBV probe containing the entire genome on samples obtained in the United States revealed that the former gave a small number of false negatives. Further comparative testing of samples obtained from Japan exhibited an even greater significance of false negatives. These results indicated that it was possible that the relatively short oligonucleotide probes used in the hybridization assay were not binding to some of the samples due to strain variation at the DNA level (i.e., the samples included non-adw subtypes that varied enough to affect the binding with the oligonucleotide probes).

Accordingly, the capture and amplifier probe sets were redesigned from a computer comparison of the nucleotide sequences of the nine HBV subtypes reported in

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Gene Bank. These probes (subtype non-specific, SN) are of varying length and were permitted to contain up to a 32-fold level of degeneracy (multiple nucleotides in various positions). Their 3' sequences are shown in FIG. 9. As shown, these sequences were extended with the 20-mers LLA2C (for the amplifier set) and XTI (for the capture set).

Assays using these redesigned probes were compared with assays using the probes of Example 3 (adw) and dot blot. (The microtiter dish MTD format using a five-site unit multimer was used in the sandwich hybridizations.) The results are given below.

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	<u>Genomic Dot Blot</u>	<u>adw MTD</u>	<u>SN MTD</u>
H 35	+++	++	+++
H 36	++	++	+++
5 H 33	++	++	+++
H 34	++	+	+++
H 32	+++	-	+++
10 H 18	++	-	++
H 21	++	-	++
H 22	++	-	+++
H 23	++	-	+++
15 H 24	++	-	++
H 25	++	-	++
H 27	++	-	++
H 28	++	-	++
20 H 31	++	-	+
3	+++	-	++
5	++	-	+
6	++	-	+
25 15	+++	-	+++
17	+++	-	++
42	++	-	+++
48	++	-	+++
30 adr	+	-	+
adw	+	+	+

35 As mentioned, the adw assay negative samples were positive in the SN assay and genomic dot blot-- indicating the samples are subtype other than adw. A sample of known adr subtype was employed as a control.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in nucleic acid chemistry, biochemical assays, and related fields are intended to be within the scope of the following claims.

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Claims

1. A solution sandwich DNA hybridization for detecting HBV DNA in an analyte comprising:

5 (a) contacting the analyte under hybridizing conditions with an excess of (i) a set of amplifier probe oligonucleotides each comprising a first segment having a nucleotide sequence complementary to a segment of the HBV genome and a second segment having a nucleotide sequence  
10 complementary to an oligonucleotide unit of a nucleic acid multimer and (ii) a set of capture probe oligonucleotides each comprising a first segment having a nucleotide sequence complementary to another segment of the HBV genome and a second segment having a nucleotide sequence  
15 complementary to an oligonucleotide bound to a solid phase;

(b) contacting under hybridizing conditions the product of step (a) with the oligonucleotide bound to the solid phase;

20 (c) thereafter separating materials not bound to the solid phase;

(d) contacting under hybridizing conditions the solid phase complex product of step (c) with the nucleic acid multimer, said multimer comprising (i) at least one  
25 oligonucleotide unit that is complementary to the second segments of the amplifier probe oligonucleotides and (ii) a multiplicity of second oligonucleotide units that are complementary to a labeled oligonucleotide;

(e) removing unbound multimer;

30 (f) contacting under hybridizing conditions the solid phase complex product of step (e) with the labeled oligonucleotide;

(g) removing unbound labeled oligonucleotide;

and

35 (h) detecting the presence of label in the solid phase complex product of step (g), characterized in that

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the first segments of the amplifier probe oligonucleotides and capture probe oligonucleotides have sequences that are complementary to consensus HBV ds region sequences based on a multiplicity of HBV subtypes.

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2. The solution sandwich DNA hybridization of claim 1 wherein the first segments of the amplifier probe oligonucleotides have the following sequences:

10 T G A C T G ( C G ) C G A T T G G T ( G A ) G A G G C A G G ( A C ) G G A G G ,  
C T T G ( A T ) ( C T ) G G G ( G A ) T T G A A G T C C C A A T C T G G A T T ,  
G T T G C G T C A G C A A A C A C T T G G C A ( C G ) A G A C C ( A T ) ,  
T A A G T T G G C G A G A A A G T ( G A ) A A A G C C T G ( T C ) T T ( A C ) ,  
G C A G C A A A ( G A ) C C C A A A A G A C C C A C A A ( T G ) ( T A ) C ( T G ) ( T C ) ,  
15 A T G T A T A C C C A ( G A ) A G A C A ( A G ) A A G A A A A T T G G T ,  
T A G A G G A C A A A C G G G C A A C A T A C C T T G ( A G ) T A ,  
G A T G A G G C A T A G C A G C A G G A T G A A G A G G A A ,  
G A T A A A A C G C C G C A G A C A C A T C C A G C G A T A ,  
G G A C A A ( A G ) T T G G A G G A C A ( G A ) G A G G T T G G T G A G ,  
20 T T G G A G G T T G G G G A C T G C G A A T T T T G G C C A ,  
C C A C C A C G A G T C T A G A C T C T G ( C T ) G G T A T T G T ,  
G A T T C T T G T C A A C A A G A A A A C C C C G C C T G ,  
C A C G A G ( C A ) A G G G G T C C T A G G A A T C C T G A T G T ,  
C A G G G T T T A C T G T T C C ( T G ) G A A C T G G A G C C A C ,  
25 C A G G G T C C C C A G T C C T C G ( A C ) G ( A G ) A G A T T G A C G ,  
C C G T T G C C G A G C A A C G G G G T A A A G G T T ( C A ) A ( G T ) ,  
G G T T G C G T C A G C A A A C A C T T G G C A ( G C ) A G A C C ,  
A G T T C C G C A G T A T G G A T C G G C A G A ( C G ) G A G C C ,  
C C A G A C C ( T G ) ( C G ) C T G C G A G C A A A C A A G C ( T G ) G C T ,  
30 C A G T T G G C A G ( C T ) A C A ( C G ) C C T A G C A G C C A T G G A ,  
G G G A C G T A ( A G ) A C A A A G G A C G T C C C G C G ( A C ) A G G ,  
C G A G A ( A C G ) G G G T C G T C C G C ( A G ) G G A T T C A G C G C C ,  
C C G C G T A A A G A G A G G T G C G C C C C G T G G T C G ,  
A C A C G G ( T A ) C C G G C A G A T G A G A A G G C A C A G A C ,  
35 C ( T G ) C C A T G C ( A G T ) A C G T G C A G A G G T G A A G C G ,

-51-

CAAGAGTCCTCTT (AG) TGTAAGACCTTGGGCA,  
 AACA (AC) ACAGTCTTTGAAGTA (TG) GCCTCAAGG,  
 CTAATCTCCTCCCCCA (AG) CTCCTCCAGTC (CT),  
 TGCCTACAGCCTCCTA (AG) TACAAAGA (CT) C (AT),  
 5 GACATG (AT) ACA (AT) GAGATGATTAGGCAGAGG (GT),  
 CTTTATA (CA) GG (AG) TC (GA) ATGTCCATGCCCAA,  
 AAAA (AC) GAGAGTAACTCCACAG (AT) (AT) GCTCAA,  
 AGGAGTGCGAATCCACACTCC (AG) AAAGA (GCT) AC,  
 TAA (GA) GATAGGGGCATTTGGTGGTCT (AG) TA (GA) GC,  
 10 TCGTCTAACAACAGTAGT (CT) TCCGGAAGTGT,  
 GCTGTAG (CA) TCTTGTTCCCAAGAATATGGTG, and  
 (TC) GCCCTGAGCCTG (AC) GGGCTCCACCCCAAAA, wherein  
 the parentheses indicate two-fold degenerative positions  
 and the first segments of the capture probe  
 15 oligonucleotides have the following sequences:

CTTGGCCCCCAATACCACATCATCCATATA,  
 GAAAGCCAAACAGTGGGGGAAAGCCCTACG,  
 CACTGAACAAATGGCACTAGTAACTGAGC,  
 20 GAGAAACGG (AG) CTGAGGCC (AC) CTCCCATAGG,  
 (GC) CGAAAGCCCAGGA (CT) GATGGGATGGGAATA,  
 CGAGGCGAGGGAGTTCTTCTTCTAGGGGAC,  
 TCTTCTGCGACGCGGCGAT (GT) GAGA (TC) CT (GT) CGT,  
 GG (AG) ATACTAACATTGAGATTCCCGAGATTG,  
 25 AGCCC (CA) GTAAAGTT (TC) CC (CG) ACCTTATGAGTC, and  
 CCCAAGGCACAGCTTGGAGGCTTGAACAG, wherein the  
 parentheses indicate two-fold degenerative positions.

3. A synthetic oligonucleotide useful as a  
 30 probe in a hybridization assay for HBV selected from the  
 group consisting of oligonucleotides which includes the  
 following sequences:

TGACTG (CG) CGATTGGT (GA) GAGGCAGG (AC) GGAGG,  
 35 CTTG (AT) (CT) GGG (GA) TTGAAGTCCCAATCTGGATT,  
 GTTGCCTCAGCAAACACTTGGCA (CG) AGACC (AT),

-52-

TAAGTTGGCGAGAAAGT ( GA ) AAAGCCTG ( TC ) TT ( AC ) ,  
GCAGCAA ( GA ) CCCAAAAGACCCACAA ( TG ) ( TA ) C ( TG ) ( TC ) ,  
ATGTATACCCA ( GA ) AGACA ( AG ) AAGAAAATTGGT ,  
TAGAGGACAAACGGGCAACATACCTTG ( AG ) TA ,  
5 GATGAGGCATAGCAGCAGGATGAAGAGGAA ,  
GATAAACGCCGCAGACACATCCAGCGATA ,  
GGACAA ( AG ) TTGGAGGACA ( GA ) GAGGTTGGTGAG ,  
TTGGAGGTTGGGGACTGCGAATTTTGGCCA ,  
CCACCACGAGTCTAGACTCTG ( CT ) GGTATTGT ,  
10 GATTCTTGTCAACAAGAAAACCCCGCGT ,  
CACGAG ( CA ) AGGGTCTTAGGAATCCTGATGT ,  
CAGGGTTTACTGTTCC ( TG ) GAACTGGAGCCAC ,  
CAGGGTCCCAGTCCTCG ( AC ) G ( AG ) AGATTGACG ,  
CCGTTGCCGAGCAACGGGGTAAAGGTT ( CA ) A ( GT ) ,  
15 GGTGCGTCAGCAAACACTTGGCA ( GC ) AGACC ,  
AGTCCGCAGTATGGATCGGCAGA ( CG ) GAGCC ,  
CCAGACC ( TG ) ( CG ) CTGCGAGCAAACAAGC ( TG ) GCT ,  
CAGTTGGCAG ( CT ) ACA ( CG ) CCTAGCAGCCATGGA ,  
GGGACGTA ( AG ) ACAAAGGACGTCCCGCG ( AC ) AGG ,  
20 CGAGA ( ACG ) GGGTCGTCCGC ( AG ) GGATTCAGCGCC ,  
CCGCGTAAAGAGAGGTGCGCCCCGTGGTGC ,  
ACACGG ( TA ) CCGGCAGATGAGAAGGCACAGAC ,  
C ( TG ) CCATGC ( AGT ) ACGTGCAGAGGTGAAGCG ,  
CAAGAGTCCTCTT ( AG ) TGTAAGACCTTGGGCA ,  
25 AACA ( AC ) ACAGTCTTTGAAGTA ( TG ) GCCTCAAGG ,  
CTAATCTCCTCCCCA ( AG ) CTCCTCCCAGTC ( CT ) ,  
TGCTACAGCCTCCTA ( AG ) TACAAAGA ( CT ) C ( AT ) ,  
GACATG ( AT ) ACA ( AT ) GAGATGATTAGGCAGAGG ( GT ) ,  
CTTTATA ( CA ) GG ( AG ) TC ( GA ) ATGTCCATGCCCCAAA ,  
30 AAAA ( AC ) GAGAGTAACTCCACAG ( AT ) ( AT ) GCTCCAA ,  
AGGAGTGCGAATCCCACTCC ( AG ) AAAGA ( GCT ) AC ,  
TAA ( GA ) GATAGGGGCATTTGGTGGTCT ( AG ) TA ( GA ) GC ,  
TCGTCTAACAACAGTAGT ( CT ) TCCGGAAGTGT ,  
GCTGTAG ( CA ) TCTTGTTCCTCAAGAATATGGTG ,  
35 ( TC ) GCCCTGAGCCTG ( AC ) GGGCTCCACCCCAAAA ,

-53-

CTTGGCCCCAATACCACATCATCCATATA,  
 GAAAGCCAAACAGTGGGGGAAAGCCCTACG,  
 CACTGAACAAATGGCACTAGTAAACTGAGC,  
 GAGAAACGG (AG) CTGAGGCC (AC) CTCCCATAGG,  
 5 (GC) CGAAAGCCCAGGA (CT) GATGGGATGGGAATA,  
 CGAGGCGAGGGAGTTCTTCTTCTAGGGGAC,  
 TCTTCTGCGACGCGGCGAT (GT) GAGA (TC) CT (GT) CGT,  
 GG (AG) ATACTAACATTGAGATTCCCGAGATTG,  
 AGCCC (CA) GTAAAGTT (TC) CC (CG) ACCTTATGAGTC, and  
 10 CCCAAGGCACAGCTTGGAGGCTTGAACAG, wherein the  
 parentheses indicate two-fold degenerative positions.

4. A set of synthetic oligonucleotides useful  
 as amplifier probes in the solution-phase sandwich assay  
 15 of claim 1 comprising oligonucleotides which include the  
 following sequences:

TGA CTG (CG) CGATTGGT (GA) GAGGCAGG (AC) GGAGG,  
 CTTG (AT) (CT) GGG (GA) TTGAAGTCCCAATCTGGATT,  
 20 GTTGCCTCAGCAAACACTTGGCA (CG) AGACC (AT) ,  
 TAAGTTGGCGAGAAAGT (GA) AAAGCCTG (TC) TT (AC) ,  
 GCAGCAA (GA) CCCAAAAGACCCACAA (TG) (TA) C (TG) (TC) ,  
 ATGTATACCCA (GA) AGACA (AG) AAGAAAATTGGT,  
 TAGAGGACAAACGGGCAACATACCTTG (AG) TA,  
 25 GATGAGGCATAGCAGCAGGATGAAGAGGAA,  
 GATAAAACGCCGAGACACATCCAGCGATA,  
 GGACAA (AG) TTGGAGGACA (GA) GAGGTTGGTGAG,  
 TTGGAGGTTGGGGACTGCGAATTTGGCCA,  
 CCACCACGAGTCTAGACTCTG (CT) GGTATTGT,  
 30 GATTCTTGTCACAAGAAAACCCCGCCTG,  
 CACGAG (CA) AGGGGTCCTAGGAATCCTGATGT,  
 CAGGGTTTACTGTTCC (TG) GAACTGGAGCCAC,  
 CAGGGTCCCCAGTCCTCG (AC) G (AG) AGATTGACG,  
 CCGTTGCCGAGCAACGGGGTAAAGGTT (CA) A (GT) ,  
 35 GGTTCGCTCAGCAAACACTTGGCA (GC) AGACC,  
 AGTTCCGAGTATGGATCGGCAGA (CG) GAGCC,

-54-

CCAGACC (TG) (CG) CTGCGAGCAAACAAGC (TG) GCT,  
 CAGTTGGCAG (CT) ACA (CG) CCTAGCAGCCATGGA,  
 GGGACGTA (AG) ACAAAGGACGTCCCGCG (AC) AGG,  
 CGAGA (ACG) GGGTCGTCCGC (AG) GGATTCAGCGCC,  
 5 CCGCGTAAAGAGAGAGGTGCGCCCCGTGGTGC,  
 ACACGG (TA) CCGGCAGATGAGAAGGCACAGAC,  
 C (TG) CCATGC (AGT) ACGTGCAGAGGTGAAGCG,  
 CAAGAGTCTCTT (AG) TGTAAGACCTTGGGCA,  
 AACA (AC) ACAGTCTTTGAAGTA (TG) GCCTCAAGG,  
 10 CTAATCTCCTCCCCCA (AG) CTCTCCAGTC (CT),  
 TGCCTACAGCCTCCTA (AG) TACAAAGA (CT) C (AT),  
 GACATG (AT) ACA (AT) GAGATGATTAGGCAGAGG (GT),  
 CTTTATA (CA) GG (AG) TC (GA) ATGTCCATGCCCAAA,  
 AAAA (AC) GAGAGTAACTCCACAG (AT) (AT) GCTCCAA,  
 15 AGGAGTGCGAATCCACACTCC (AG) AAAGA (GCT) AC,  
 TAA (GA) GATAGGGGCATTTGGTGGTCT (AG) TA (GA) GC,  
 TCGTCTAACAACAGTAGT (CT) TCCGGAAGTGT,  
 GCTGTAG (CA) TCTTGTTCCCAAGAATATGGTG, and  
 (TC) GCCCTGAGCCTG (AC) GGGCTCCACCCCAAAA, wherein  
 20 the parentheses indicate two-fold degenerative positions.

5. A set of synthetic oligonucleotides useful  
 as capture probes in the solution phase hybridization  
 assay of claim 1 comprising oligonucleotides which include  
 25 the following sequences:

CTTGGCCCCAATACCACATCATCCATATA,  
 GAAAGCCAAACAGTGGGGAAAGCCCTACG,  
 CACTGAACAAATGGCACTAGTAAACTGAGC,  
 30 GAGAAACGG (AG) CTGAGGCCC (AC) CTCCCATAGG,  
 (GC) CGAAAGCCCAGGA (CT) GATGGGATGGGAATA,  
 CGAGGCGAGGGAGTTCTTCTTCTAGGGGAC,  
 TCTTCTGCGACGCGCGAT (GT) GAGA (TC) CT (GT) CGT,  
 GG (AG) ATACTAACATTGAGATTCCCGAGATTG,  
 35 AGCCC (CA) GTAAAGTT (TC) CC (CG) ACCTTATGAGTC, and  
 CCCAAGGCACAGCTTGGAGGCTTGAACAG, wherein the  
 parentheses indicate two-fold degenerative positions.



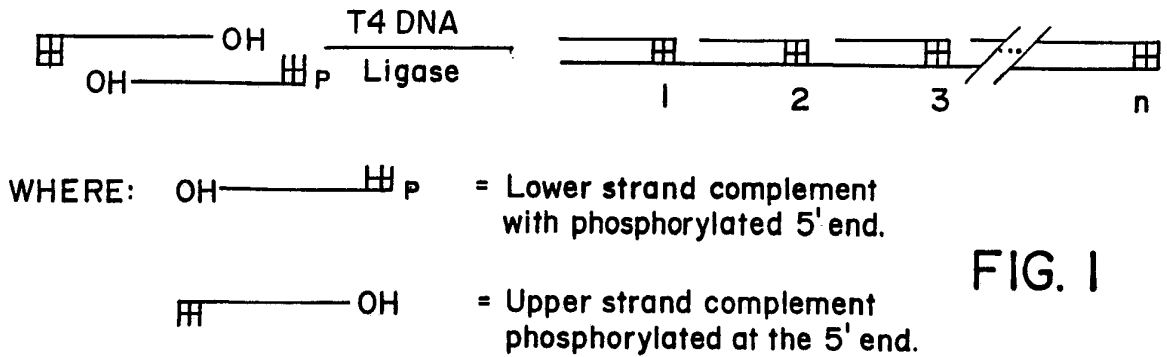


FIG. 1

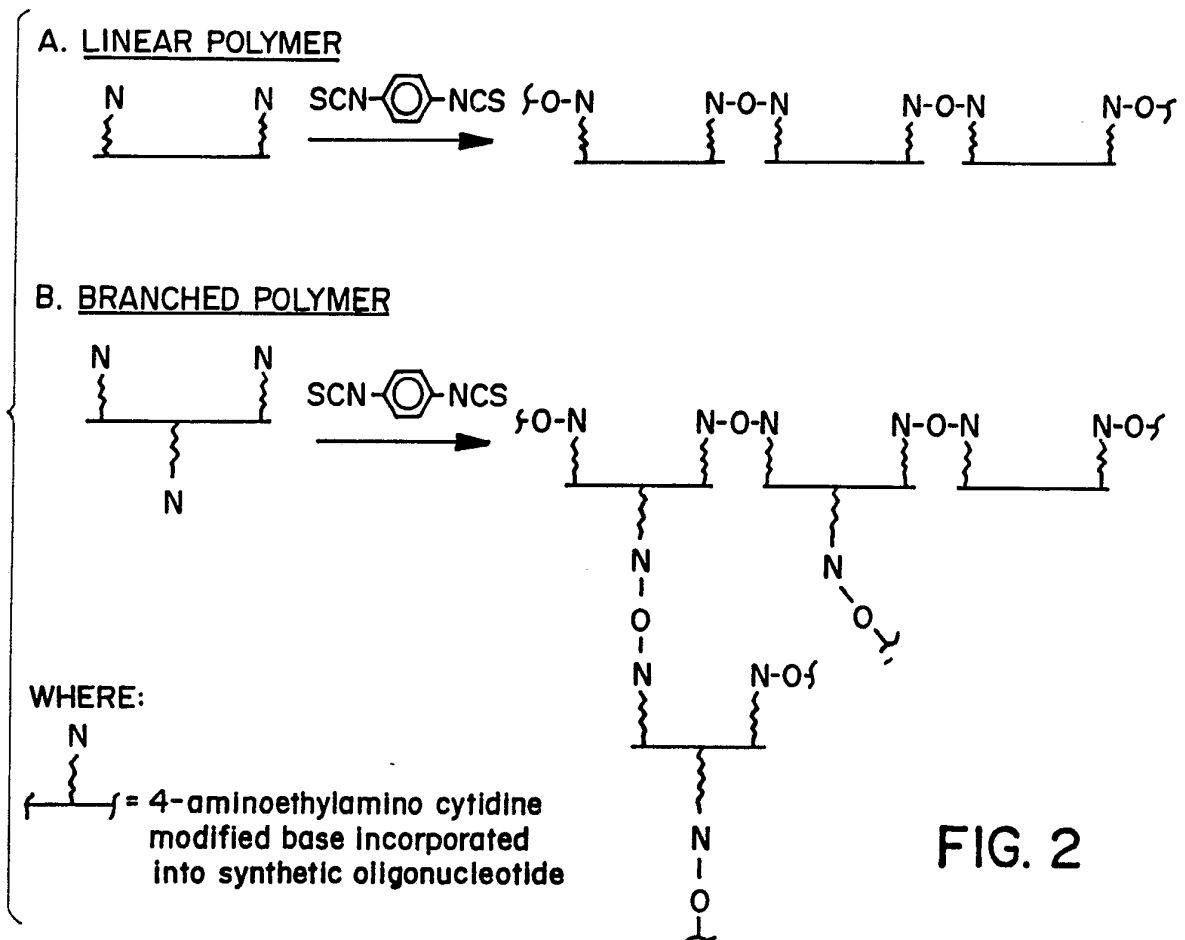
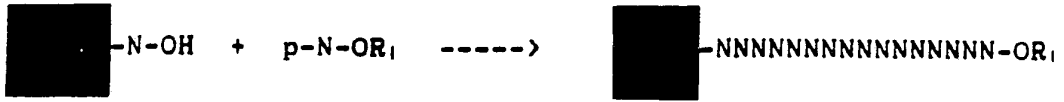


FIG. 2

A. Linear synthetic DNA.



B. Branched DNA, comb structures.

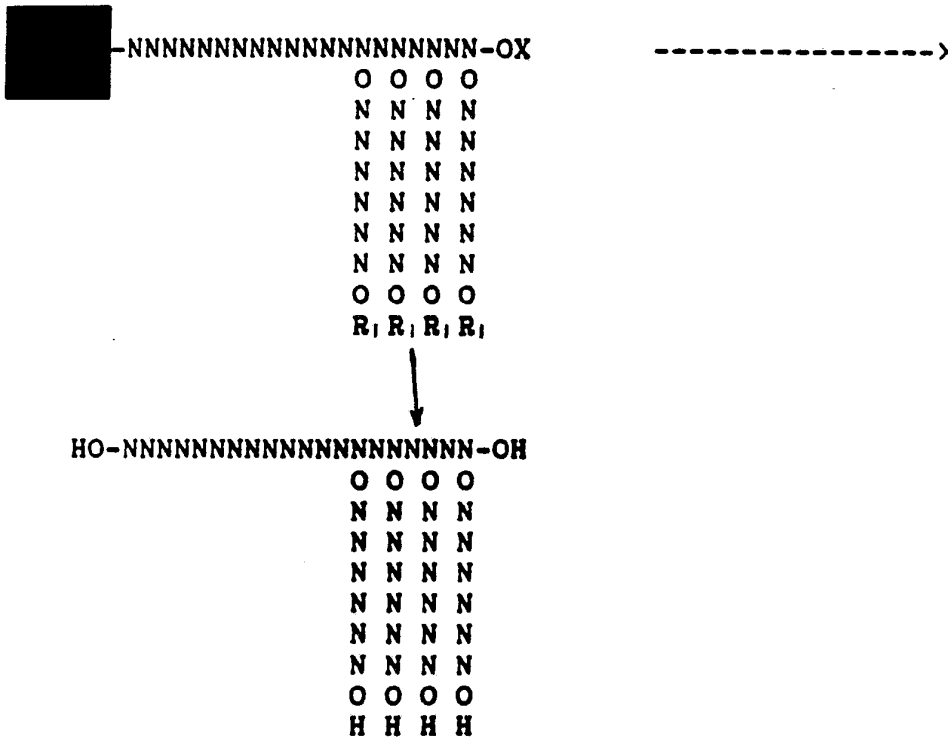
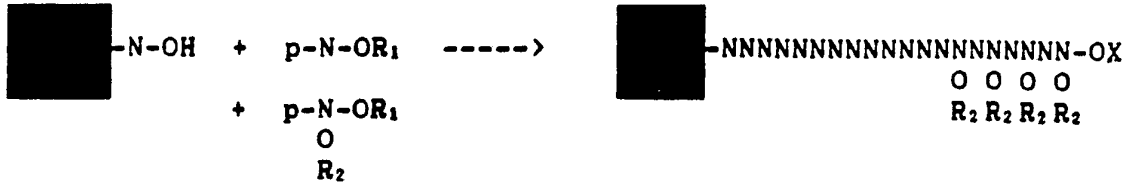


FIG. 3 - 1

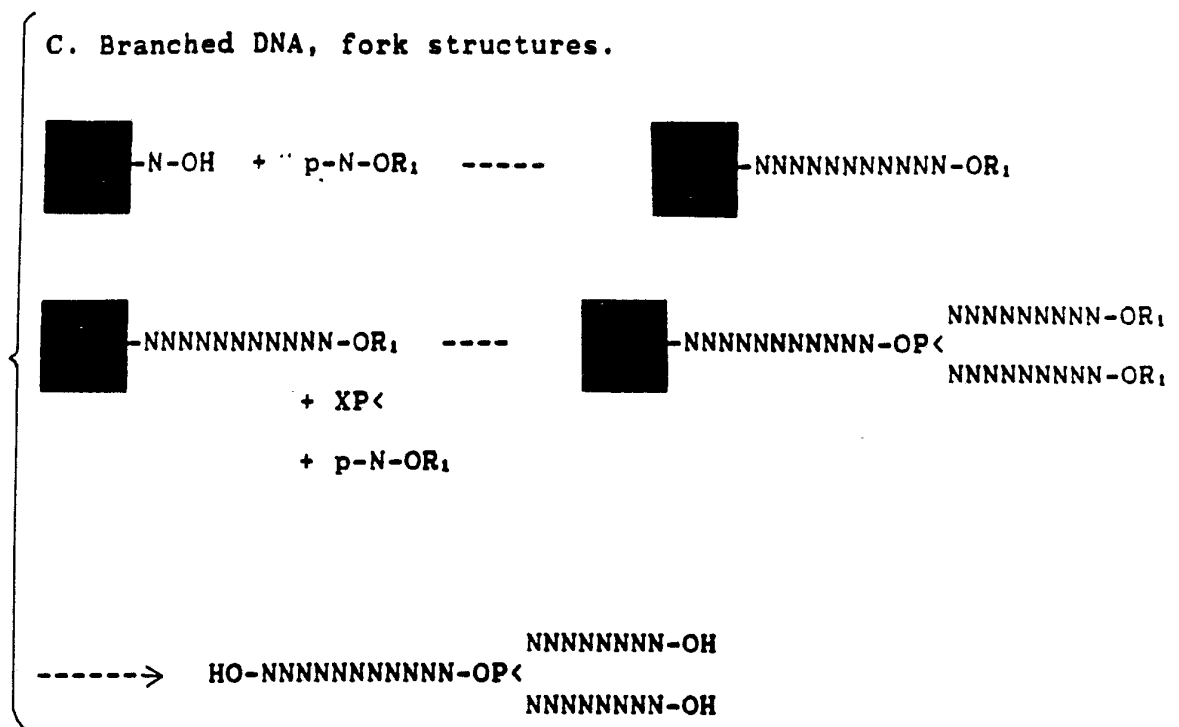
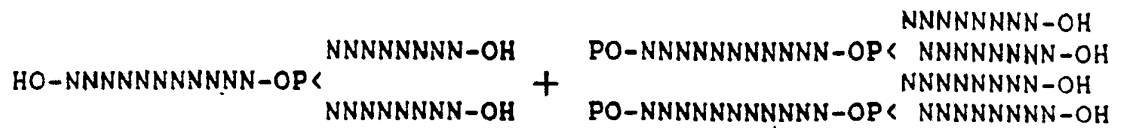


FIG. 3-2

D. Multiple Forks (through solution assembly).



- 1) Splicing linker used for T<sub>4</sub> ligase.
- 2) Chemical ligation also possible.

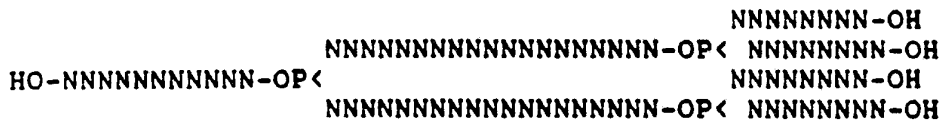
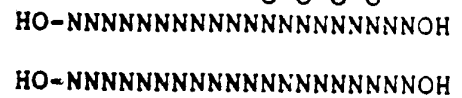
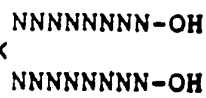


FIG. 3-3

E. Forked comb structures.



- 1) Splicing linker used for T4 ligase assembly.
- 2) Chemical assembly also possible.

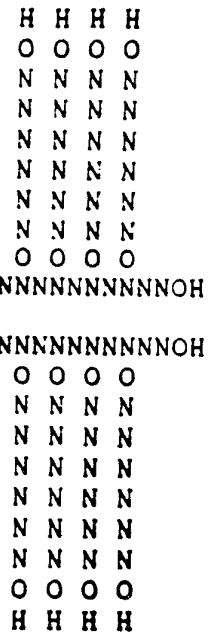
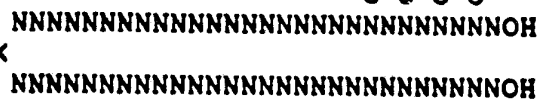


FIG. 3-4

F. Multiple Comb Structure

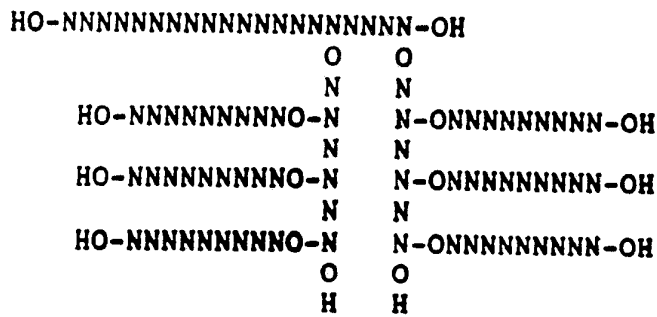
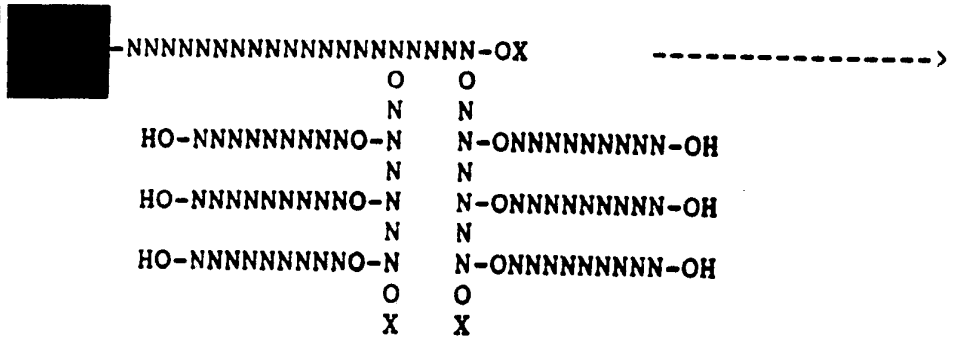
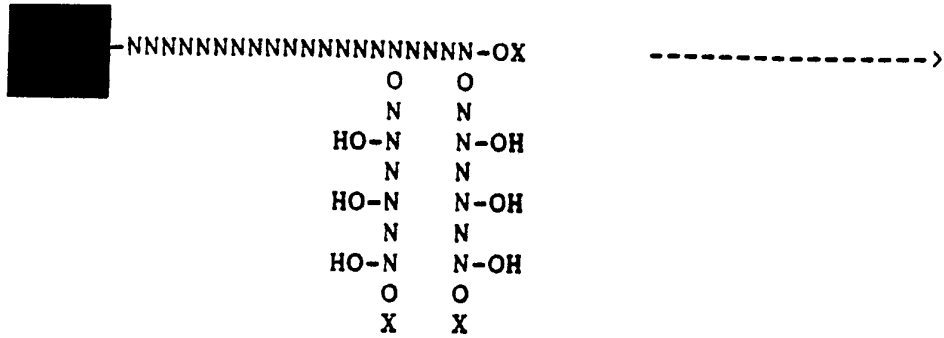


FIG. 3-5

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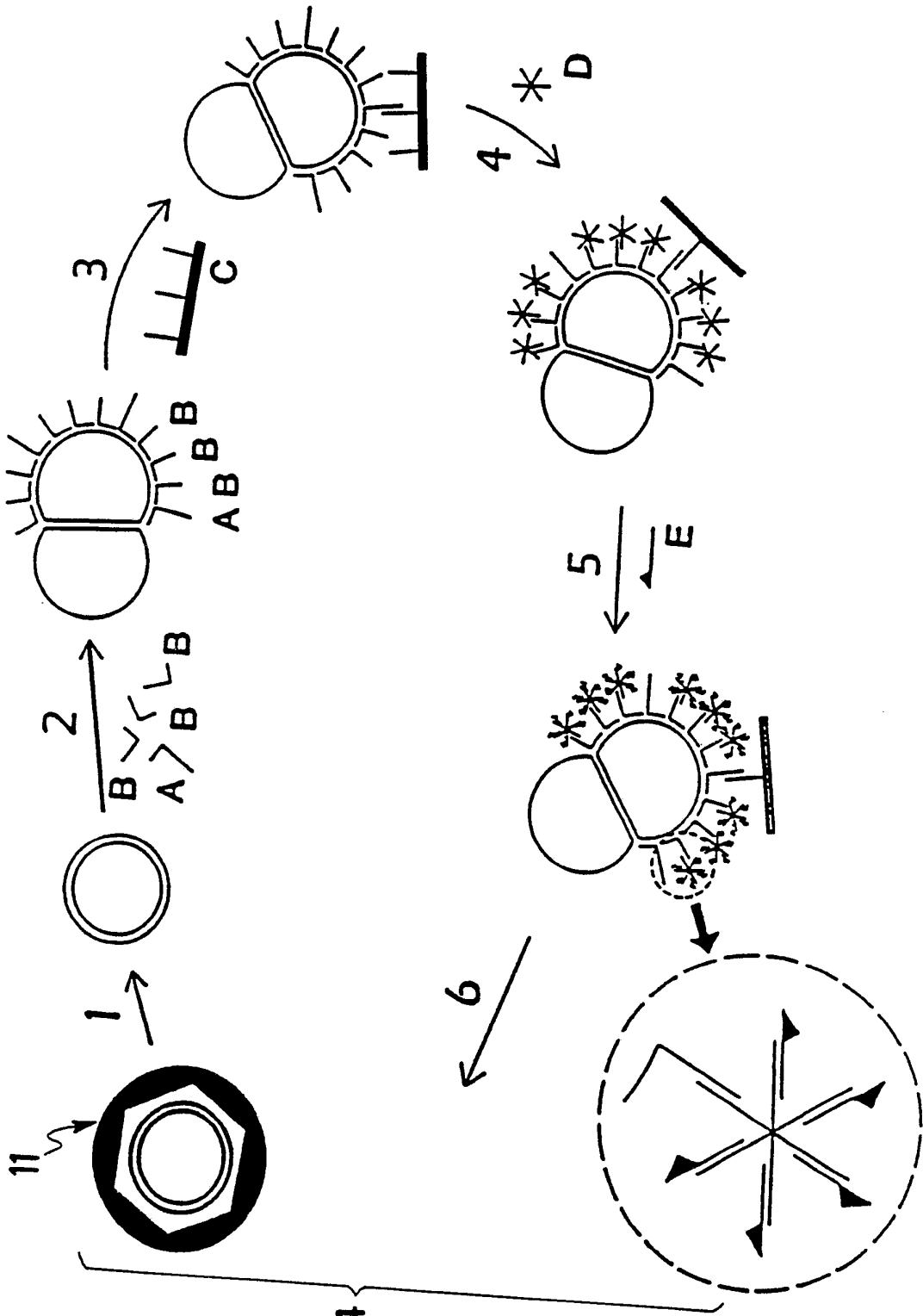


FIG. 4

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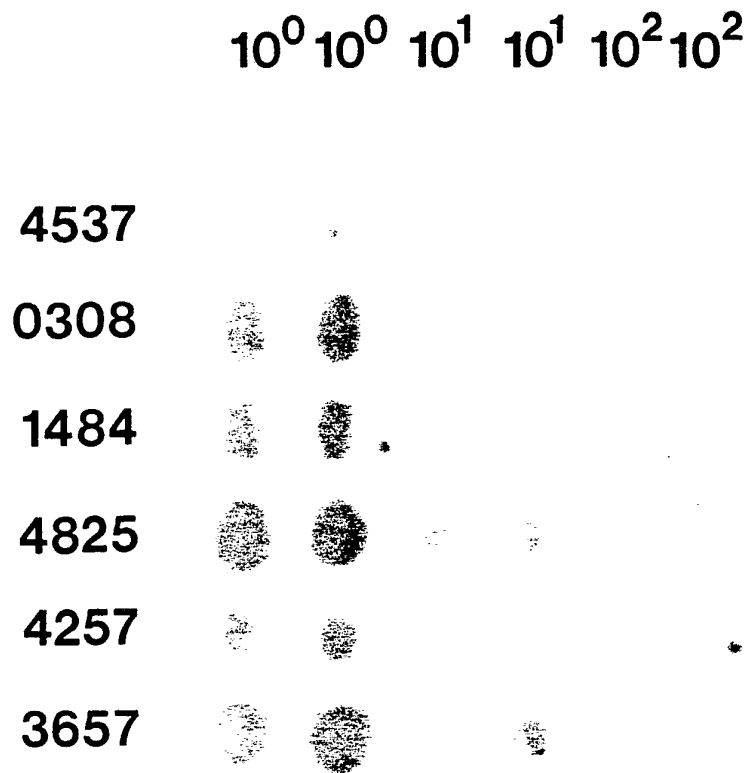
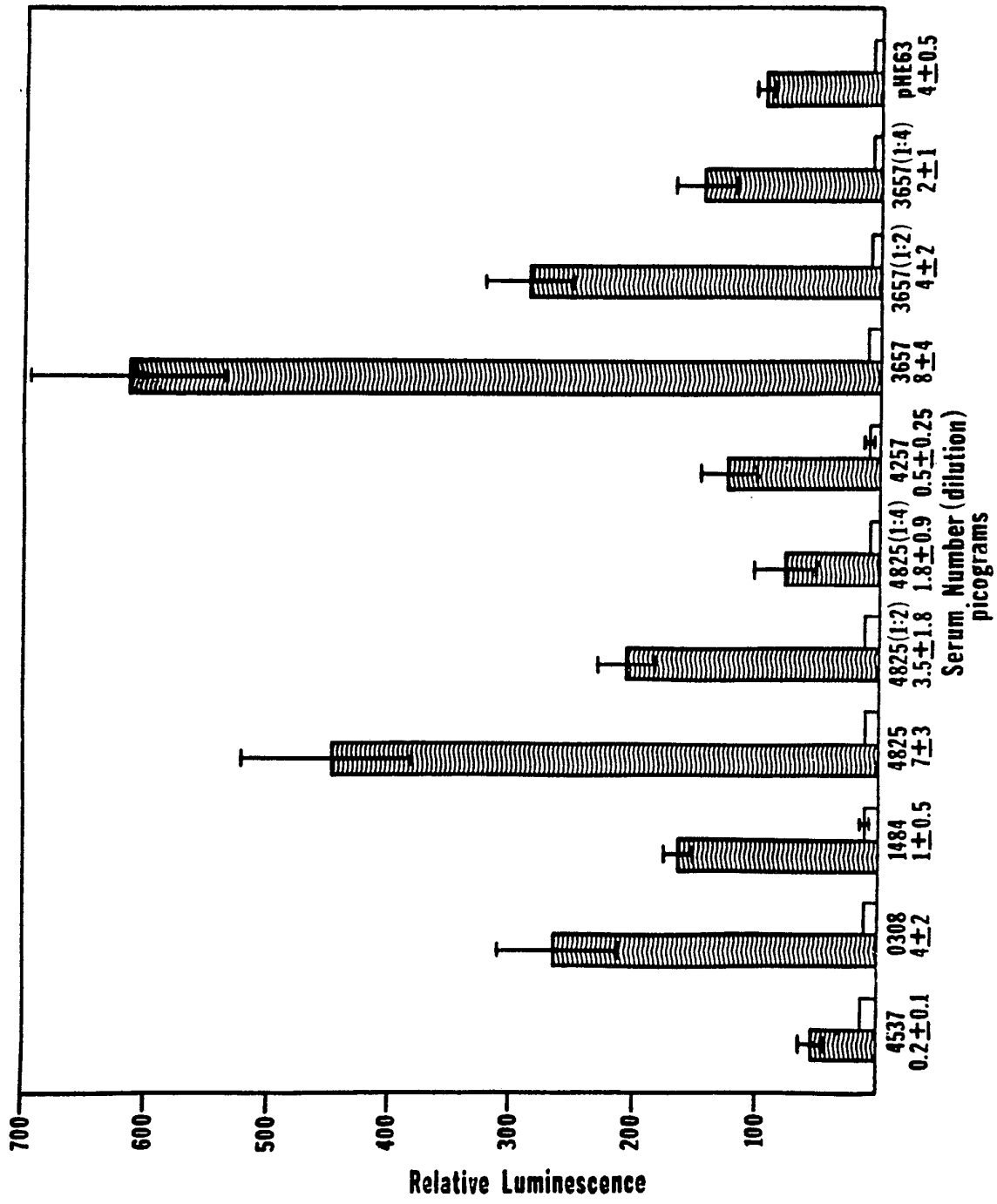


FIG. 5



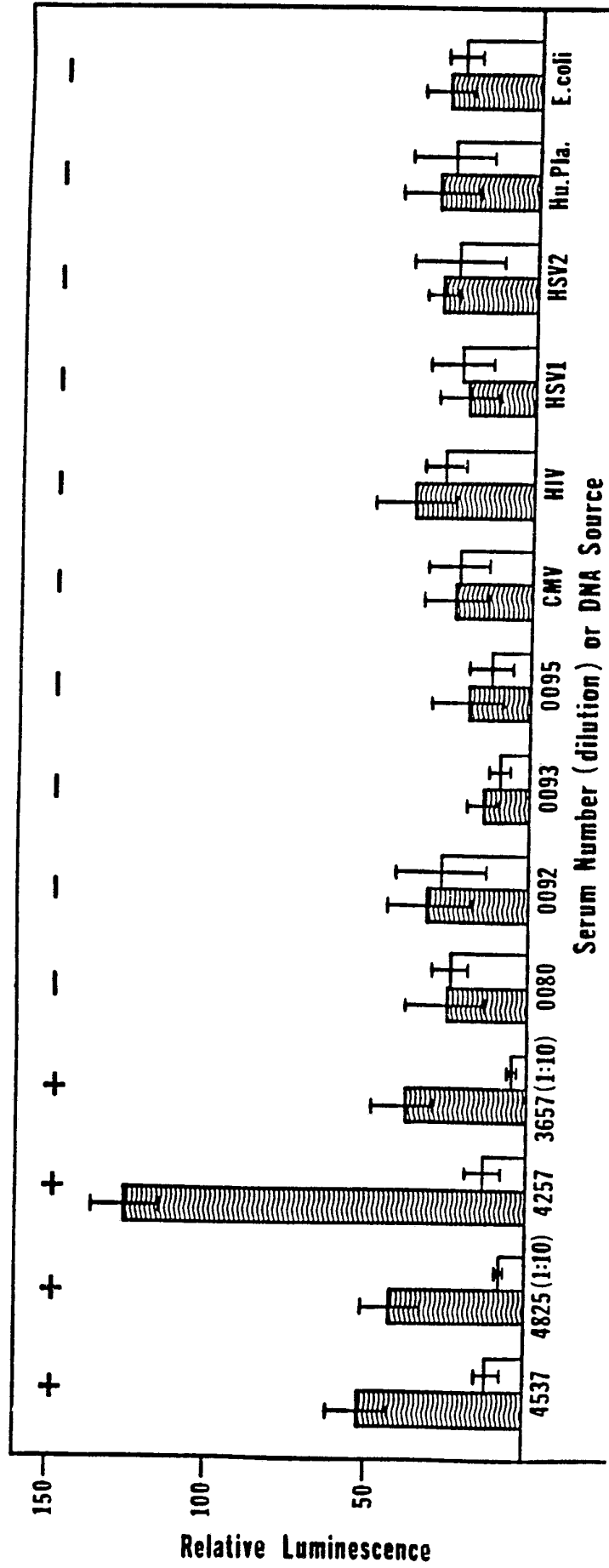
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FIG. 6



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FIG. 7-1



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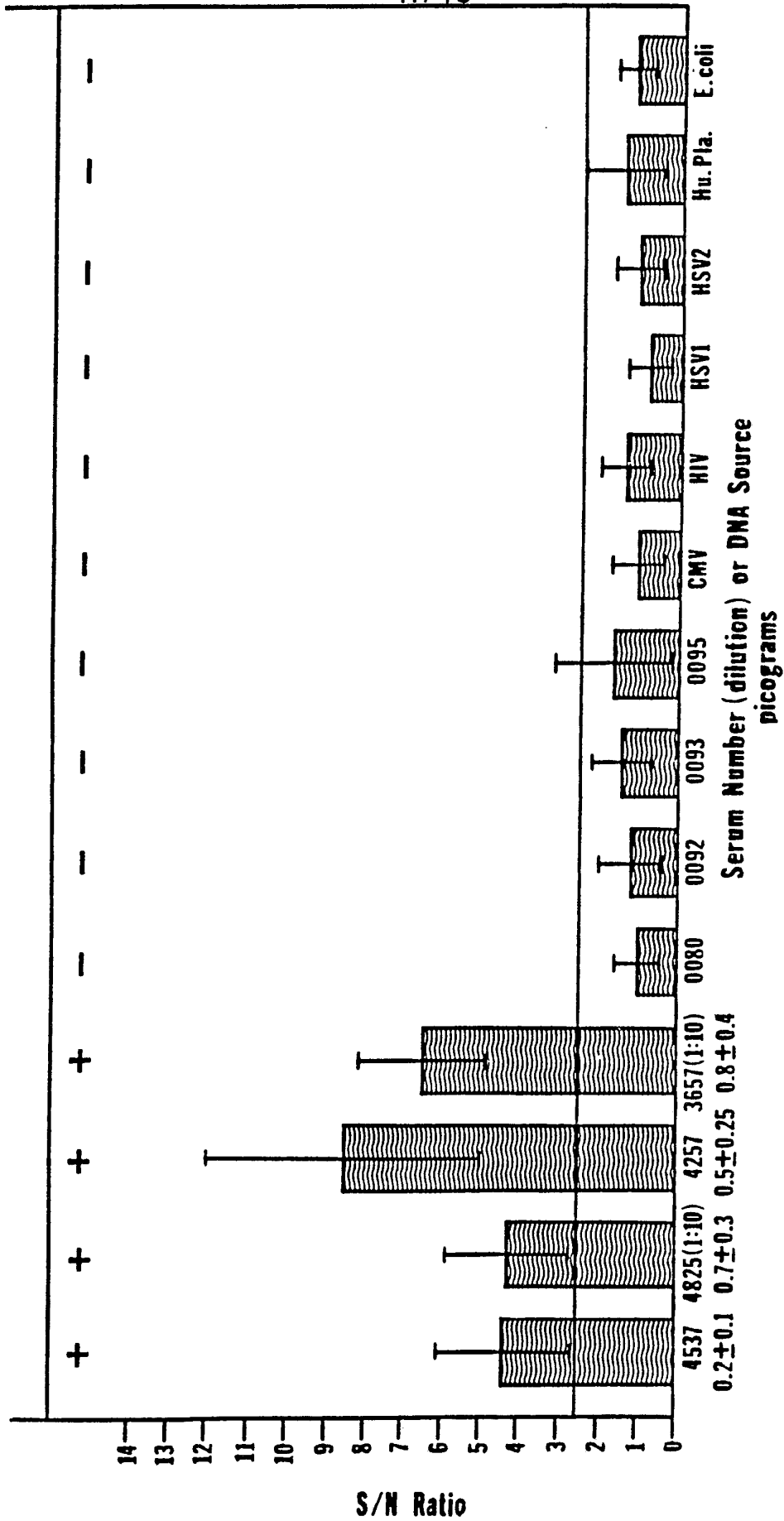


FIG. 7-2

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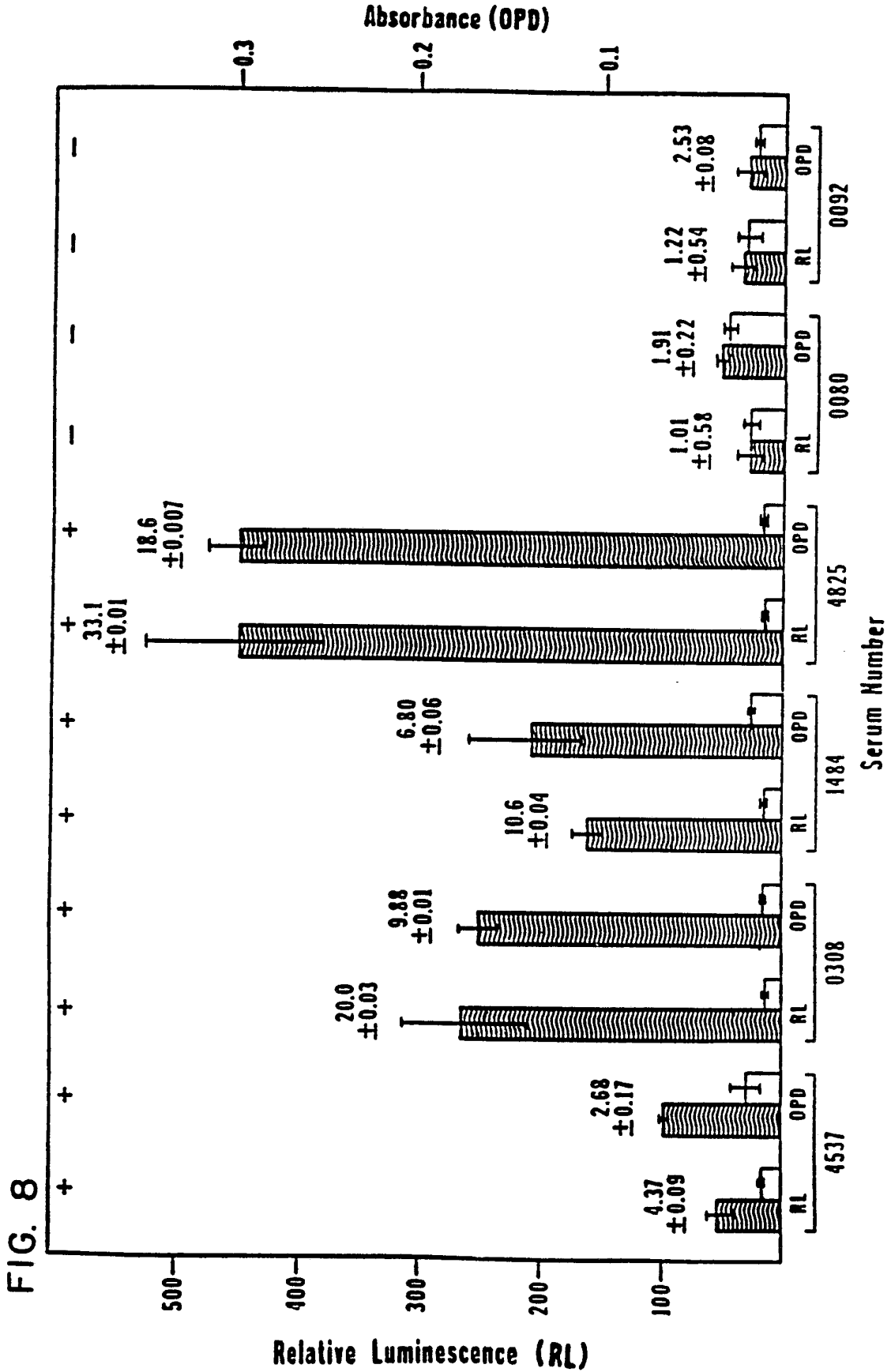


FIG. 8

## FIG. 9-1

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:HBV.LLA2C.53  
GATTCTTGTCAACAAGAAAAACCCCGCCTGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.52  
CACGAG [CA] AGGGGTCCCTAGGAATCCTGATGTTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.51  
CAGGGTTTACTGTTCC [TG] GAACTGGAGCCACTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.71  
CAGGGTCCCCAGTCCTCG [AC] G [AG] AGATTGACGTTAGGCAGAGGACCCGTGTC

:HBV.LLA2C.72  
CCGTTGCCGAGCAACGGGGTAAAGGTT [CA] A [GT] TTAGGCATAGGACCCGTGTC

:HBV.LLA2C.73  
GGTTGCGTCAGCAAACACTTGGCA [GC] AGACCTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.74  
AGTCCGCAGTATGGATCGGCAGA [CG] GAGCCTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.75  
CCAGACC [TG] [CG] CTGCGAGCAAAACAAGC [TG] GCTTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.76  
CAGTTGGCAG [CT] ACA [CG] CCTAGCAGCCATGGATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.77  
GGGACGTA [AG] ACAAAGGACGTCCCGCG [AC] AGGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.78  
CGAGA [ACG] GGGTCGTCCGC [AG] GGATTCAGCGCCTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.79  
CCGCGTAAAGAGAGGTGCGCCCCGTGGTCGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.80  
ACACGG [TA] CCGGCAGATGAGAAGGCACAGACTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.81  
C [TG] CCATGC [AGT] ACGTGCAGAGGTGAAGCGAAGTTAGGCATAGGACCCGTGTC

:HVB.LLA2C.82  
CAAGAGTCCTCTT [AG] TGTAAGACCTTGGGCATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.83  
AACA [AC] ACAGTCTTTGAAGTA [TG] GCCTCAAGGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.84  
CTAATCTCCTCCCCA [AG] CTCCTCCAGTC [CT] TTAGGCATAGGACCCGTGTC

:HBV.LLA2C.85  
TGCCTACAGCCTCCTA [AG] TACAAAGA [CT] C [AT] TTTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.D44  
GACATG [AT] ACA [AT] GAGATGATTAGGCAGAGG [GT] TTAGGCATAGGACCCGTGTC

:HBV.LLA2C.D46  
CTTTATA [CA] GG [AG] TC [GA] ATGTCCATGCCCCAAATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.D47  
AAAA [AC] GAGAGTAACTCCACAG [AT] [AT] GCTCCAATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.86

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AGGAGTGCGAATCCCACTCC [AG] AAAGA [GCT] ACTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.87

TAA [GA] GATAGGGGCATTTGGTGGTCT [AG] TA [GA] GCTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.88

TCGTCTAACAACAGTAGT [CT] TCCGGAAGTGTTTAGGCATAGGACCCGTGTC

:HBV.XT1.89

CGAGGCGAGGGAGTTCTTCTTCTAGGGGACCTTCTTTGGAGAAAGTGGTG

:HBV.XT1.90

TCTTCTGCGACGCGGCGAT [GT] GAGA [TC] CT [GT] CGTCTTCTTTGGAGAAAGTGGTG

:HBV.XT1.D13

GG [AG] ATACTAACATTGAGATTCCCGAGATTGCTTCTTTGGAGAAAGTGGTG

:HBV.XT1.D14

AGCCC [CA] GTAAAGTT [TC] CC [CG] ACCTTATGAGTCCTTCTTTGGAGAAAGTGGTG

:HBV.LLA2C.91

GCTGTAG [CA] TCTTGTTCCCAAGAATATGGTGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.92

[TC] GCCCTGAGCCTG [AC] GGGCTCCACCCCAAATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.45

ACCCAAGGCACAGCTTGGAGGCTTGAACAGTTAGGCATAGGACCCGTGTC

FIG. 9-2

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## FIG. 9-3

:HBV.LLA2C.70

TGACTG [CG] CGATTGGT [GA] GAGGCAGG [AC] GGAGGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.69

CTTG [AT] [CT] GGG [GA] TTGAAGTCCCAATCTGGATTTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.67

TAAGTTGGCGAGAAAGT [GA] AAAGCCTG [TC] TT [AC] TTAGGCATAGGACCCGTGTC

:HBV.LLA2C.66

GCAGCAAA [GA] CCCAAAAGACCCACAA [TG] [TA] C [TG] [TC] TTAGGCATAGGACCCGTGTC

:HBV.LLA2C.65

ATGTATACCCA [GA] AGACA [AG] AAGAAAATTGGTTTAGGCATAGGACCCGTGTC

:HBV.XT1 .64

CTTGGCCCCAATACCACATCATCCATATACTTCTTTGGAGAAAGTGGTG

:HBV.XT1 .63

GAAAGCCAAACAGTGGGGGAAAGCCCTACGCTTCTTTGGAGAAAGTGGTG

:HBV.XT1 .62

CACTGAACAAATGGCACTAGTAAACTGAGCCTTCTTTGGAGAAAGTGGTG

:HBV.XT1 .61

GAGAAACGG [AG] CTGAGGCC [AC] CTCCATAGGCTTCTTTGGAGAAAGTGGTG

:HBV.XT1 .60

[GC] CGAAAGCCCAGGA [CT] GATGGGATGGGAATACTTCTTTGGAGAAAGTGGTG

:HBV.LLA2C.59

TAGAGGACAAACGGGCAACATACCTTG [AG] TATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.58

GATGAGGCATAGCAGCAGGATGAAGAGGAATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.57

GATAAAACGCCGCAGACACATCCAGCGATATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.56

GGACAA [AG] TTGGAGGACA [GA] GAGGTTGGTGAGTTAGGCATAGGACCCGTGTC

:HBV.LLA2C.55

TTGGAGGTTGGGGACTGCGAATTTTGGCCATTAGGCATAGGACCCGTGTC

:HBV.LLA2C.54

CCACCACGAGTCTAGACTCTG [CT] GGTATTGTTTAGGCATAGGACCCGTGTC

SUBSTITUTE SHEET

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02049

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) <sup>3</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): C12Q 1/68		
U.S.C1.: 435/6		
II. FIELDS SEARCHED		
Minimum Documentation Searched <sup>4</sup>		
Classification System	Classification Symbols	
U.S.	435/6, 91, 810 536/27	436/501 935/23, 78, 88
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>5</sup>		
III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>14</sup>		
Category *	Citation of Document, <sup>16</sup> with indication, where appropriate, of the relevant passages <sup>17</sup>	Relevant to Claim No. <sup>18</sup>
Y	US, A, 4,711,955 (WARD ET AL.) 08 December 1987, see entire document.	1, 2, 4, 5
Y	US, A, 4,358,535 (FALKOW ET AL.) 09 November 1982, see the abstract.	1-5
Y	US, A, 4,766,062 (DIAMOND ET AL.) 23 August 1988, see especially Figure 6.	1, 2, 4, 5
P, X	US, A, 4,868,105 (URDEA ET AL.) 19 September 1989, see entire document.	1-5
Y	WO, A, 86/07387 (SNITMAN ET AL.) 18 December 1986, see entire document.	1, 2, 4, 5
Y	WO, A, 87/03622 (SCHNEIDER ET AL.) 18 June 1987, see entire document.	1, 2, 4, 5
Y	EP, A, 0,204,510 (COLLINS) 10 December 1986, see entire document.	1, 2, 4, 5
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>15</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"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</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"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.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search <sup>2</sup>		Date of Mailing of this International Search Report <sup>2</sup>
07 September 1990		<b>25 SEP 1990</b>
International Searching Authority <sup>1</sup>		Signature of Authorized Officer <sup>20</sup>
ISA/US		Ardin Marschel



**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

X	GENE, Volume 61, issued 1987, M.S. Urdea et al., "A Novel method for the rapid detection of specific nucleotide sequences in crude biological samples without blotting or radioactivity; application to the analysis of hepatitis B virus in human serum", pages 253-264, see entire document.	1-5
X	EP, A, 0,225,807 (URDEA ET AL.) 16 June 1987, see entire document.	1-5

**V.  OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.  Claim numbers ..... because they relate to subject matter<sup>1</sup> not required to be searched by this Authority, namely:
2.  Claim numbers ..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out<sup>1</sup>, specifically:
3.  Claim numbers ..... , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI.  OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>**

This International Searching Authority found multiple inventions in this international application as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4.  As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- The additional search fees were accompanied by applicant's protest.
- No protest accompanied the payment of additional search fees.