HYBRIDIZATION AND MISMATCH DISCRIMINATION USING Oligonucleotides Conjugated to Minor Groove Binders

Conjugates between a minor groove binding molecule, such as the trimer of 1,2-dihydro-(3H)-pyrrolo[3,2-ε]indole-7-carboxylate (CDPI), and an oligonucleotide form unusually stable hybrids with complementary target sequences, in which the tethered CDPI group resides in the minor groove of the duplex. These conjugates can be used as probes and primers. Due to their unusually high binding affinity, conjugates as short as 8-mers can be used as amplification primers with high specificity and efficiency. MGB conjugation also increases the discriminatory power of short oligonucleotides, providing enhanced detection of nucleotide sequence mismatches by short oligonucleotides. The MGB-conjugated probes and primers described herein facilitate various analytic and diagnostic procedures, such as amplification reactions, PCR, detection of single-nucleotide polymorphisms, gene hunting, differential display, fluorescence energy transfer, hydrolyzable probe assays and others; by allowing the use of shorter oligonucleotides, which have higher specificity and better discriminatory power.
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HYBRIDIZATION AND MISMATCH DISCRIMINATION USING OLIGONUCLEOTIDES CONJUGATED TO MINOR GROOVE BINDERS

TECHNICAL FIELD

The present invention is in the field of molecular biology. More specifically, the invention is in the field of assays that utilize oligonucleotides as primers or hybridization probes.

BACKGROUND

In many analytic, diagnostic and experimental systems in modern biology, oligonucleotides are used in procedures that require that they base pair (i.e., hybridize) with a nucleic acid sequence that is complementary to the oligonucleotide. This hybridization process may be used to directly detect a sequence in a nucleic acid molecule (i.e., probing), to initiate synthesis at a specific sequence (i.e., priming), or to block synthesis by inhibiting primer extension (i.e., clamping). In all these procedures, the technique relies on the formation of a nucleic acid duplex (or hybrid) based on the principle that the duplex will form only if the two strands are complementary over a significant portion of their lengths. Complementarity is determined by the formation of specific hydrogen bonds between the nucleotide bases of the two strands such that only the base pairs adenine-thymine, adenine-uracil and guanine-cytosine form hydrogen bonds, giving sequence specificity to the double stranded duplex. In a duplex formed between an oligonucleotide and another nucleic acid molecule, the stability of the duplex is a function of its length, the number of specific (i.e., A-T, A-U and G-C) hydrogen bonded base pairs, and the base composition (ratio of guanine-cytosine to adenine-thymine or adenine-uracil base pairs), since guanine-cytosine pairs provide a greater contribution to the stability of the duplex than do adenine-thymine or adenine-uracil pairs.

Usually, the relative stability of a duplex is measured experimentally by heating the duplex in solution until the strands of the duplex separate. The quantitative stability of a duplex is expressed by the temperature at which one-half the base pairs have dissociated, commonly known as the “melting temperature” or Tm. In practice, this is usually measured by monitoring the ultraviolet absorbance of a solution of nucleic acid while the temperature is increased and denoting the Tm as the temperature at half the maximal absorbance at 260
nm (since an increase in absorbance at 260 nm accompanies the dissociation of the two strands of a duplex).

Essentially all procedures involving analysis of a target nucleic acid sequence require a hybridization step, either to determine directly if the complement of a known sequence (the probe) is present in a sample or to initiate synthesis (prime) from a specific sequence. Control of the specificity of the hybridization step is key to successful and accurate nucleic acid analysis. In most cases, exact matching between the sequence of the probe or primer and the sequence of its target is required. Nevertheless, in some cases, the analytical approach requires the stabilization of a probe or primer in a duplex that is not a perfect match. Therefore, techniques and material that can be used to control hybridization procedures such that it is possible, on the one hand, to obtain only perfectly matched duplexes and, under alternate conditions, to stabilize mismatched duplexes, would extend the use of oligonucleotides and allow analytical and experimental procedures that are now very difficult or unreliable.


Each of these procedures requires hybridization, to a target sequence, of an oligonucleotide primer from whose 3’ terminus synthesis is initiated. The ability of an oligonucleotide to serve as a primer depends upon the stability of the duplex it forms with its template, especially at its 3’ terminus. The ability of an oligonucleotide to serve as a
unique, specific primer depends upon the stability of the duplex its forms with its perfect complement and, conversely, on the lack of stability of a duplex including one or more noncomplementary (i.e., mismatched) base pairs. Current priming methods rely on the use of oligonucleotides sufficiently long to form stable duplexes at temperatures necessary or convenient for extension. However, longer oligonucleotides are more prone to mismatch pairing than shorter oligonucleotides. Further, specific information may restrict the use of longer oligonucleotides.

To give one example, many methods involving oligonucleotides utilize some type of amplification technology, often based on a polymerase chain reaction (PCR). See, for example, U.S. Patent Nos. 4,683,195; 4,683,202; and 4,800,159. PCR has become an exceptionally powerful tool in molecular biology, but certain factors limit its versatility. Because PCR involves multiple cycles of DNA denaturation, elevated temperatures are usually required, making the use of a thermophilic polymerizing enzyme necessary to avoid the inconvenience of supplying fresh polymerizing enzyme at each cycle. However, at the elevated temperatures optimal for activity of a thermophilic polymerase and required for denaturation, oligonucleotides shorter than about 20 nucleotides (20-mers) do not form hybrids that are stable enough to serve as primers for polymerase-catalyzed elongation. Consequently, current PCR-based techniques generally require primers at least 20 nucleotides in length to form hybrids that will be stable at the temperatures and stringencies commonly used for PCR. Saiki (1989) In Erlich, H.A. (ed.), PCR Technology: Principles and Applications for DNA Amplification. Stockton Press, pp. 7-16.

In another example, mRNA "indexing" requires priming from the 3' end of a messenger RNA (mRNA) molecule or from a cDNA made from the mRNA. Kato et al., supra. This technique employs separate populations of oligo-dT-containing primers, each additionally containing an extension of one to approximately three nucleotides adjacent to the oligo A sequence on the 5' side of the oligo A. The objective is to cause synthesis of specific segments of DNA corresponding to the 3' end of each mRNA (determined by the oligo A sequence) but separated into specific populations, determined by the specific base at positions 1 to (approximately) 3, upstream of the oligo A. If each primer is used in a separate reaction, separate populations of cDNAs are generated, each of which is a subset of the total mRNA. These can be used to analyze cellular expression. This procedure is usually combined with PCR, by including a second primer in each separate reaction. The
practicability of this method is limited by the necessity to use sufficiently long oligo thymidylate, complementary to the oligo A, to stabilize the first primer. This can result in stabilization of mismatches within the one to three specific bases at the 3' end of the primer. In a population of primer and templates, these mismatches allow synthesis of improperly primed, and therefore misleading cDNA molecules leading to incorrect indexing of mRNA. Alternatively, some primers are insufficiently stable to prime efficient synthesis; consequently, the extension products they would have generated are underrepresented in the population, again leading to incorrect indexing of the corresponding mRNA. Short primers that are stable at elevated temperatures commonly used for PCR, but that form only perfect duplexes (i.e., do not prime mismatches) would increase the utility of this technique.

Important new techniques, such as gene hunting and differential display, would also benefit from the use of shorter primers. In some cases, short primers are essential for these methods. In gene hunting, a family of amplified transcripts shares a short degenerate sequence that specifies a conserved peptide motif, and this priming sequence is necessarily limited in length. Tung et al., supra. Stockton press, pp. 99-104. In differential display, complete representation of a transcript pool is sought, and this is optimally achieved by priming with 6-mers. The impracticality of using such short primers necessitates the use of longer degenerate ODNs. Liang et al., supra. However, long degenerate ODNs may not provide an accurate representation of the complexity of a mRNA population, since mispriming can generate non-specific products, and inefficient hybridization of the primer can lead to underrepresentation of certain transcripts. Buchner et al. (1995) Stat. Mol. Biol. 8:12-14. Application of longer oligonucleotides to viral diagnostics are limited, because amplification of a common sequence from multiple strains can be complicated by the presence of genomic variability. Smits et al. (1992) J. Gen. Virol. 73:3263-3268. Again, shorter primers are desirable, since the shorter the sequence used for priming, the less likely that it will encompass a region characterized by genomic variability.

In addition to priming, oligonucleotide hybridization is used in several techniques to probe nucleic acid sequences. In general, these assays require that the probes form perfectly-matched duplexes with target sequences. These assays are usually based on one of three schemes: 1) The probe or target is labeled (e.g., with a radioactive isotope, a fluorescent dye or a reactive compound), the nucleic acids are placed under hybridization
conditions following hybridization, the non-hybridized labeled material is removed and the remaining label is quantitated. 2) The probe is specifically labeled and placed with the target DNA under hybridization conditions, following hybridization, the hybridized probe is detected by virtue of a property unique to a duplex containing the probe such as susceptibility to a duplex-specific nuclease (e.g., U.S. Patent 5,210,015), 3) fluorescence generated by interaction of a dye with duplex DNA (Wittwer et al. (1997) BioTechniques 22:130-138) or separating a fluorophore from a quenching dye by the extension of the probe as a result of hybridization.

A method that could be used in essentially all these types of nucleic acid hybrid detection systems to enhance the distinction between exact duplexes and duplexes with one or more mismatched base pairs would be a very useful tool in specific nucleic acid sequence determination and clearly be valuable in clinical diagnosis, genetic research and forensic laboratory analysis.

For example, many diseases are associated with known inherited polymorphisms or mutations. Many of these are due to single nucleotide changes and, to be useful, a genetic assay based on hybrid formation must be able to distinguish between a hybrid with all base pairs matched and one with a single mismatch. A group of single base differences at certain points in the sequences of human DNA called single nucleotide polymorphisms have been determined to be stably inherited genetic markers (Schaeffer et al. (1998) Nature Biotechnology 16:33). These markers can be associated with ancestral populations and in some cases can be associated with characteristics such as disease susceptibility or response to environmental factors such as chemicals, drugs, etc. Although these polymorphisms can theoretically be discovered by the tedious process of gene sequencing, their use as genetic markers associated with a phenotype in, for example, medical practice or research, necessitates a screening or typing system that is capable of analyzing DNA from tens to hundreds of individuals. This process will not easily be accommodated by current methods of DNA sequencing. Single nucleotide polymorphism analysis thus represents an additional field in which there exists a need for a reliable method for distinction of single base differences in DNA sequences by a process such as hybridization.

Various additional assays that involve oligonucleotide priming are known in the art. These include, but are not limited to, assays that utilize the nuclease activity of a polymerase enzyme to release label from a probe hybridized to an extension product (see,
for example, U.S. Patent No. 5,210,015), and assays in which hybridization of two or more oligonucleotides to adjacent sites on a target nucleic acid results in interactions between the oligonucleotides, such as, for example, fluorescence resonance energy transfer. See, for example, Stavrianopoulos et al., U.S. Patent No. 4,868,103; and Heller et al., European Patent Publication 070,685. These techniques are also limited by the length of the oligonucleotide that can be used for efficient hybridization and/or priming. The ability to use shorter oligonucleotides would therefore be beneficial in these procedures and, indeed, in any application that involves hybridization of an oligonucleotide to a target nucleic acid.

Chemical modification of short oligonucleotides has been attempted, with an eye toward improving hybrid stability while retaining effective priming ability. Certain modifications, such as N3'→P5' phosphoramidates (Gryaznov et al. (1994) J. Am. Chem. Soc. 116:3143-3144) and peptide (Nielsen et al. (1994) Bioconjugate Chem. 5:3-7) or guanidine (Dempcy et al. (1995) Proc. Natl. Acad. Sci. USA, 92:6097-6101) linkages, have been shown to enhance hybrid stability. However, such modified oligonucleotides are non-extendible, because they lack a 3'-OH group, and are therefore unable to serve as primers. Other hybrid-stabilizing modifications that have not been investigated with respect to their ability to support primer extension are 2'-modified sugars (Monia et al. (1993) J. Biol. Chem. 268:14514-14522; Sproat et al. (1993) In Crooke, S.T. and Lebleu, B. (eds), Antisense Research and Applications. CRC Press, Boca Raton, FL, pp. 352-362), conjugated intercalating agents (Asseline et al. (1984) Proc. Natl. Acad. Sci. USA 81:3297-3301) and substituted bases such as 2-aminoadenine (Lamm et al. (1991) Nucleic Acids Res. 19:3193-3198) or C5 propynyl pyrimidines (Wagner et al. (1993) Science 260:1510-1513). Thus, the need remains for a method of modifying short oligonucleotides so that they form more stable hybrids, such that the modification will not interfere with the ability of the oligonucleotides to serve as primers.

A further shortcoming in the use of oligonucleotides as probes and primers is the difficulty of obtaining specificity such as single nucleotide mismatch discrimination using oligonucleotide probes and/or primers. In many cases, it is necessary to distinguish target sequences which differ by a single nucleotide and, in some cases, it would be desirable to do so using oligonucleotides. That is, it would be useful to have a given oligonucleotide which is able to hybridize to a target sequence with which it is complementary along its entire length (a perfect hybrid or perfect match), but which, under identical stringency
conditions, will not hybridize to a target sequence that is non-complementary to the oligonucleotide at a single nucleotide residue (a single-nucleotide mismatch). Unfortunately, this type of single nucleotide mismatch discrimination is possible only when fairly short (for example, <20 mer) oligonucleotides are used. The disadvantage of using such short oligonucleotides is that they hybridize weakly, even to a perfectly complementary sequence, and thus must be used under conditions of reduced stringency. If it were possible to achieve single nucleotide mismatch discrimination under conditions of high stringency (such as those under which most amplification reactions are conducted), improvements in speed and efficiency would accrue in techniques such as allele-specific oligonucleotide hybridization, single nucleotide polymorphism analysis, and functional genomics, to name just a few.

DISCLOSURE OF THE INVENTION

The present invention relates to a covalently bound minor groove binder (MGB)-oligonucleotide conjugate which includes an oligonucleotide having a plurality of nucleotide units, a 3'-end and a 5'-end, and a minor groove binder moiety covalently attached to at least one of said nucleotides or said ends. The minor groove binder is typically attached to the oligonucleotide through a linking group. The minor groove binder moiety is a radical of a molecule having a molecular weight of approximately 150 to approximately 2000 Daltons which molecule binds in a non-intercalating manner into the minor groove of double stranded DNA, RNA or hybrids thereof with an association constant greater than approximately $10^3$ M$^{-1}$.

The present invention also relates to the use of MGB-oligonucleotide conjugates as hybridization probes and primers for analytical and diagnostic, as well as therapeutic (anti-sense and anti-gene) purposes.

It has now been discovered that conjugation of a minor groove binder (MGB) to an oligonucleotide (ODN) dramatically increases the stability of the hybrid formed between the oligonucleotide and its target. Increased stability (*i.e.*, increased degree of hybridization) is manifested in a higher melting temperature ($T_m$; the temperature at which half of the base pairs have become unpaired) of hybrid duplexes formed by such MGB-oligonucleotide conjugates, compared to those formed by an unconjugated oligonucleotide of identical length and sequence. This effect is particularly pronounced for
short oligonucleotides (e.g., less than about 21 nucleotides in length) and makes possible,  
for the first time, the use of short oligonucleotides as probes and primers, under high  
stringency conditions. Conjugation of an oligonucleotide with a MGB, with its attendant  
increase in hybrid stability, does not adversely affect the ability of the conjugated  
oligonucleotide to serve as a primer. Therefore, it is now possible, using the methods and  
compositions of the present invention, to use shorter oligonucleotides than previously  
required in techniques in which hybridization is required, such as polymerase chain  
reactions and hydrolyzable probe assays, which are generally conducted at high stringency,  
due to the use of high temperatures and thermophilic enzymes.

In addition to increased duplex stabilization, MGB-oligonucleotide conjugates  
retain the heightened sensitivity to sequence mismatch that is characteristic of  
unconjugated short oligonucleotides with low melting temperatures. Thus, conjugation to a  
MGB endows very short oligonucleotides (e.g., oligonucleotides containing less than about  
21 nucleotides) with greater specificity, by endowing them with the potential to form  
hybrids having a stability characteristic of much longer oligonucleotides, while retaining  
the ability to discriminate between sequences differing by a single nucleotide. Use of short  
oligonucleotides at high stringency now becomes possible, using MGB-oligonucleotide  
conjugates.

The use of MGB-oligonucleotide conjugates as probes and primers provides  
improvements in speed, sensitivity and versatility to a variety of assays involving  
hybridization of oligonucleotides. Such assays are well-known in the art and include, but  
are not limited to, single nucleotide mismatch detection, in situ hybridization, polymerase  
chain reaction (PCR, see U.S. Patents 4,683,202; 4,683,195 and 4,800,159), allele-specific  
oligonucleotide (ASO) hybridization (Huang et al. (1992) Acta Haematol. 88:92-95),  
detection of single-nucleotide polymorphism (Mullis and Faloona; Meth. Enzymol., vol.  
ed, Birkhauser, Basel, pp. 21-28), random amplification of polymorphisms in DNA  
740), DNA amplification fingerprinting (Caetano-Anollés et al. (1991) Biotechnology  
9:553-557), assays involving fluorescence energy transfer, assays involving release of label  

by exonuclease-mediated hydrolysis of a hybridized oligonucleotide probe, assays involving ligation of two or more oligonucleotides, etc.

All patents, patent applications and publications mentioned herein, either supra or infra, are hereby incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the structure of 1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate, also known as CDPI3. Also shown are the structures of the linkers used for conjugation of CDPI3 to the 5' and 3' ends of oligonucleotides.

Figure 2 shows a comparison of unmodified and MGB-conjugated 16-mer, 12-mer and 10-mer oligonucleotides as PCR primers. Conjugated oligonucleotides contained a 5'-CDPI3 moiety. The indicated pair of primers was used to amplify a segment of single-stranded M13mp19 DNA according to the procedure described in Example 1. PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

In Figure 2A, MGB-oligonucleotide conjugates (lanes 1-4) were compared to unmodified oligonucleotides (lanes 5-8) as reverse primers. In all cases, the oligonucleotides were 16-mers, and the annealing temperature was 45°C. The specific pairs of oligonucleotides used as primers, and the predicted sizes of the products, were as follows. Lane 1: 4-C and I (307 nucleotides). Lane 2: 9-C and I (297 nucleotides). Lane 3: 12-C and I (217 nucleotides). Lane 4: 13-C and I (181 nucleotides). Lane 5: 4 and I (307 nucleotides). Lane 6: 9 and I (297 nucleotides). Lane 7: 12 and I (217 nucleotides). Lane 8: 13 and I (181 nucleotides). See Table 1 for the sequences and structures of the oligonucleotides, and for their location within the M13mp19 genome. Lane M comprises molecular weight markers, whose size (in nucleotides) is given to the left of the Figure.

In Figure 2B, MGB-oligonucleotide conjugates (lanes 1-4) were compared to unmodified oligonucleotides (lanes 5-8) as reverse primers. In all cases, the oligonucleotides were 16-mers, and the annealing temperature was 68°C. The specific pairs of oligonucleotides used as primers, and the predicted sizes of the products, were as follows. Lane 1: 4-C and I (307 nucleotides). Lane 2: 9-C and I (297 nucleotides). Lane 3: 12-C and I (217 nucleotides). Lane 4: 13-C and I (181 nucleotides). Lane 5: 4 and I (307 nucleotides). Lane 6: 9 and I (297 nucleotides). Lane 7: 12 and I (217 nucleotides). Lane 8: 13 and I (181 nucleotides). See Table 1 for the sequences and structures of the
oligonucleotides, and for their location within the M13mp19 genome. Lane M comprises molecular weight markers as in Figure 2A.

In Figure 2C, 10-mer (lane 1; oligonucleotides 3-C and 7-C) and 12-mer (lane 2; oligonucleotides 6-C and 2-C) MGB-oligonucleotide conjugates were used as primers and the annealing temperature was 55°C. The predicted product length was 307 nucleotides for both lanes. Lane M comprises molecular weight markers as in Figure 2A.

Figure 3 shows a comparison of unmodified and MGB-conjugated 8-mer and 6-mer oligonucleotides as PCR primers. Conjugated oligonucleotides contained a 5'-CPD1 moiety. The indicated pair of primers were used to amplify a segment of single-stranded M13mp19 DNA using a touch-down PCR protocol as described in Example 1. Products were analyzed on 8% polyacrylamide sequencing gels and visualized by silver staining.

In Figure 3A, the forward primer was a 10-mer, the reverse primers were 8-mers, and the annealing temperature was gradually decreased from 55 to 41°C. The specific primer pairs, and the predicted sizes of the products, were as follows. Lane 1: 11 and 3 (217 nucleotides). Lane 2: 8 and 3 (297 nucleotides). Lane 3: 11-C and 3-C (217 nucleotides). Lane 4: 8-C and 3-C (297 nucleotides). Lane M denotes molecular weight markers from a Hae III digest of φX174 DNA, whose sizes (in nucleotides) are given to the right of the figure.

In Figure 3B, the forward primer was a 10-mer, the reverse primers were 6-mers, and the annealing temperature was gradually decreased from 50 to 37°C. The specific primer pairs, and the predicted sizes of the products, were as follows. Lane 1: 10-C and 3-C (295 nucleotides). Lane 2: 5-C and 3-C (305 nucleotides). Lane M denotes molecular weight markers from a Hae III digest of φX174 DNA, as in Figure 3A.

Figure 4 shows the nucleotide sequence of the E. coli supF gene contained in the plasmid pSP189 (SEQ ID No.: 40), indicating the locations of the target sequences for the amplification primers (labeled “Primer 1” and “Primer 2”), the region that served as target in a hydrolyzable probe assay (labeled “15-mer”), and the single-nucleotide substitutions that were introduced into the target sequence for the experiment shown in Figure 5 (shown underneath the region labeled “15-mer”).

Figure 5 shows results of a hydrolyzable probe assay, using MGB-conjugated 15-mer probes wherein all guanine bases in the probe were substituted with the guanine
analogue ppG. The target was the *E. coli* *supF* gene. Annealing/elongation was conducted at 75°C for 20 sec per cycle.

**MODES FOR CARRYING OUT THE INVENTION**

The practice of the present invention will employ, unless otherwise indicated, conventional techniques in organic chemistry, biochemistry, oligonucleotide synthesis and modification, nucleic acid hybridization, molecular biology, microbiology, genetics, recombinant DNA, and related fields as are within the skill of the art. These techniques are fully explained in the literature. See, for example, Maniatis, Fritsch & Sambrook,


**Improved hybridization and discriminatory properties of MGB-oligonucleotide conjugates**

In many types of hybridization assay, base-pairing interactions between a probe oligonucleotide and a fully- or partially-complementary target sequence are detected, either directly (by measuring hybridized probe) or indirectly (by measuring some event that depends on probe hybridization). Modifications which improve hybridization kinetics (*i.e.*, speed up the hybridization process), change the equilibrium of the hybridization reaction to favor product (*i.e.*, increase the fraction of probe in hybrid), and/or lead to the formation of more stable hybrids, will allow more rapid, efficient and accurate hybridization assays, thereby increasing efficiency of hybridization and facilitating the use of hybridization techniques in new areas such as diagnostics and forensics. Furthermore, it is often advantageous to be able to distinguish between a perfect hybrid (or perfect match), in which every probe nucleotide is base-paired to a complementary nucleotide in the target, and an imperfect hybrid or mismatch, in which one or more probe nucleotides are not complementary to the target. For example, a hybrid between an oligonucleotide and a target nucleic sequence wherein one base in the oligonucleotide is non-complementary to
the target sequence is termed a single-nucleotide mismatch. Single-nucleotide mismatch discrimination (i.e., the ability to distinguish between a perfect match and a single-nucleotide mismatch) is extremely useful in the detection of mutations for diagnostic purposes, and in the determination of allelic single-nucleotide polymorphisms in diagnostic, therapeutic, and forensic applications. Heretofore, such single-nucleotide mismatch discrimination has been difficult to achieve, especially under conditions of high stringency.

The present invention provides, among other things, modified oligonucleotides for use as probes and primers. The oligonucleotides are modified by the covalent attachment of a minor groove binding moiety (MGB). The structure and preparation of exemplary MGBs are provided herein. A MGB-oligonucleotide conjugate having a defined sequence that is complementary to a target sequence in a second polynucleotide will form a duplex having greater hybrid strength, compared to oligonucleotide probes and primers of the prior art. A MGB-oligonucleotide conjugate whose sequence will result in a hybrid having a single-nucleotide mismatch with that of a target sequence in a second polynucleotide will form a duplex that is more easily discriminated from a perfectly-matched duplex, compared to oligonucleotide probes and primers of the prior art.

Increased hybrid stability

The strength of hybridization, or hybrid stability, between two nucleic acids, can be determined by subjecting a nucleic acid duplex to steadily increasing temperature or to a steadily increasing concentration of a denaturing agent. Ultraviolet absorbance (a measure of base-pairing) is determined as a function of temperature or concentration of denaturing agent. Absorbance increases as base pairs become unpaired and base stacking interactions are lost, and absorbance reaches a plateau when the duplex has been completely denatured. Several measures of hybridization strength can be obtained from this type of analysis. The melting temperature \((T_m)\) is commonly defined as the temperature (or concentration) at which half of the base-pairs in the duplex become unpaired. The temperature at which the change in absorbance with respect to temperature (or with respect to the concentration or intensity of denaturing agent) is at a maximum (i.e., the temperature at which \(dA/dT\) is at a maximum) is known as the \(T_{max}\) for that particular nucleic acid duplex.

Increased hybridization strength between MGB-oligonucleotide conjugates and their target sequences has several advantageous consequences. For example, short
oligonucleotides, less than about 21 nucleotides in length, are generally not suitable for use in amplification techniques such as PCR, because such techniques are conducted at elevated temperatures (usually over 70°C) that are above the T<sub>m</sub> of short oligonucleotides. However, conjugation of a MGB to such a short oligonucleotide results in an increase in hybrid stability sufficient for the MGB-oligonucleotide conjugate to achieve specific, stable hybridization at elevated temperatures, such as are used in PCR and other amplification techniques.

Another advantage of the increased hybrid stability conferred on oligonucleotides by MGB conjugation is that it is now possible to obtain hybridization to a target using an oligonucleotide containing one or more complementary sequence mismatches with the target sequence. This will make it possible to use a single defined sequence oligonucleotide as a primer or probe for a target that exhibits genetic heterogeneity. To provide just one example, MGB-oligonucleotide conjugates are useful in assays which utilize oligonucleotides for the detection of HIV. Since the HIV genome undergoes frequent mutational events, some of which lead to resistance to anti-viral therapeutics, it is possible that a mutation present in a region of the viral genome complementary to an oligonucleotide probe or primer will result in the mutant becoming undetectable in an oligonucleotide-based assay. However, MGB-oligonucleotide conjugates, by virtue of their increased hybrid stability, allow an oligonucleotide with one or more nucleotide mismatches to recognize a specific target sequence, thereby functioning as an effective probe and/or primer in systems characterized by a tendency toward frequent mutation of the target sequence. In addition, multiple subtypes of a virus, distinguished by changes in one or a few nucleotides in the target region, can be detected with a MGB-oligonucleotide conjugate having a single sequence.

Although a MGB-conjugated oligonucleotide is stabilized in its duplex relative to a non-conjugated oligonucleotide, the conjugated oligo maintains its sequence specificity, under appropriate conditions of stringency, such that mismatches can be discriminated (i.e., the stability of a duplex formed by a perfectly-matched oligonucleotide is higher than that of a mismatched duplex when the oligonucleotide is conjugated to a MGB, even though the T<sub>m</sub> of both duplexes is increased by MGB conjugation). That is to say, if a perfectly-matched oligonucleotide and an oligonucleotide differing by one nucleotide were compared (in terms of the strength of hybrids formed with a specific target sequence), the T<sub>m</sub> of
hybrids formed by both oligonucleotides would increase, but the \( T_m \) of the hybrid formed by the perfectly-matched oligonucleotide would increase by a greater extent. This effect is particularly pronounced with shorter oligonucleotides, preferably those shorter than 21 nucleotides in length and, most preferably, those shorter than 11 nucleotides in length. The result is that, even though mismatched probes form more stable hybrids when conjugated to a MGB, it is possible to distinguish a perfect match from a single-nucleotide mismatch using a MGB-oligonucleotide conjugate. In fact, the difference in \( T_m \) between a perfect match and a single-nucleotide mismatch is heightened when MGB-oligonucleotide conjugates are used as probes and/or primers (see infra).

As described herein, the present inventors have discovered that covalent attachment of a MGB to an oligonucleotide dramatically increases the stability of hybrids formed by that oligonucleotide, as measured, for example, by an increase in \( T_m \) of the hybrid. Increased hybrid stability for short oligonucleotides, as provided by the invention, provides short oligonucleotides useful as primers in various procedures involving primer extension, such as PCR, provided the appended MGB does not block the 3’-end of the oligonucleotide in a way that inhibits extension. It is shown herein (see, Example 1, infra) that MGB-oligonucleotide conjugates are indeed capable of being extended from their 3’-end by a polymerase. Thus, very short oligonucleotides (less than about 21-mers), which of themselves would form fairly unstable hybrids, are able, when conjugated to a MGB, to form hybrids with their target sequence that are stable enough to serve as primers in amplification reactions such as PCR.

There are several advantages to the use of short oligonucleotides as primers in amplification processes such as PCR. These advantages have not previously been available in procedures which are normally conducted at high temperatures using thermophilic enzymes. For example, shorter oligonucleotides are easier and less expensive to produce. Additionally, more rapid hybridization kinetics may be obtained with short oligonucleotides. Furthermore, homology searches conducted using PCR or related priming and/or amplification techniques can be based on very short regions of homology, on the order of 2-3 codons. Within this size range, single-nucleotide mismatch discrimination is maintained by MGB-oligonucleotide conjugates (see infra), making possible very sensitive searches over very limited regions of homology.
Enhanced mismatch discrimination

It is becoming increasingly important to be able to distinguish two sequences which differ from each other by a single nucleotide. Single-nucleotide polymorphisms form the molecular basis of many diseases, can determine an individual’s response to a particular therapeutic, and are useful from a forensic viewpoint. The ability to discriminate between two polynucleotides which differ by a single nucleotide is an important and valuable property of MGB-oligonucleotide conjugates. Conjugates between a MGB and a short (<21-mer) oligonucleotide retain the sequence specificity and discriminatory properties of short oligonucleotides while, at the same time, being capable of forming hybrids having stability characteristics of longer oligonucleotides. Without wishing to be bound by any particular theory, a possible explanation for the combination of these desirable properties in a MGB-oligonucleotide conjugate is as follows. Since each base-pair contributes to the stability of a hybrid; the shorter the hybrid, the greater the relative contribution of each individual base pair to the stability of a probe-target hybrid. Hence, the shorter the probe, the greater the difference in stability between a probe forming a perfect match with its target and a probe having a single base mismatch. Thus, all other things being equal, the shorter the oligonucleotide, the greater its ability to discriminate between a perfect match and a single nucleotide mismatch. However, a short oligonucleotide (even one that forms a perfect match with its target) is not able to form a stable hybrid at the elevated temperatures normally used in amplification techniques such as PCR. Thus, the potential discriminatory power of short oligonucleotides cannot be exploited in PCR and related amplification methods.

One of the effects of conjugating a MGB to a short oligonucleotide is to increase the stability (and hence the T_m) of a hybrid involving the short oligonucleotide to a degree that is compatible with use of the oligonucleotide at the elevated temperatures necessary for amplification reactions involving thermostable polymerizing enzymes. At the same time, the discriminatory properties (i.e. the heightened difference in T_m between a perfect hybrid and a single base mismatch) characteristic of short oligonucleotides are retained by the MGB-oligonucleotide conjugate under these conditions. See Examples 2, 3 and 6, infra.

Additional advantages

Since kinetics of hybridization are inversely proportional to the length of an oligonucleotide, another useful property of the MGB-oligonucleotide conjugates is that
they are capable of annealing to their target more rapidly than are longer oligonucleotides. An additional factor leading to more rapid hybridization kinetics of MGB-oligonucleotide conjugates is that the MGB is likely to serve as a nucleation site for hybridization. Furthermore, the additional free energy of binding imparted by the MGB most likely lowers the off-rate of binding between the oligonucleotide and its target. As a result, hybridization assays employing MGB-oligonucleotide conjugates can be conducted more rapidly than assays employing longer unconjugated oligonucleotides that form hybrids of comparable stability. Hence, an additional advantage provided by the novel compositions of the invention is that it is now feasible to perform hybridization analyses in situations in which time is limited. To provide but one example, one could use the methods and compositions of the invention in a surgical procedure, to investigate the molecular properties of excised tissue. Combining several advantageous properties of MGB-oligonucleotide conjugates, one would be able to test for molecular properties characteristic of the transformed state (for example, single-base mutations in oncogenes or tumor suppressor genes) in cells from a portion of resected tissue, during surgery, to guide the extent of resection. A description of the use of the methods and compositions of the invention in various techniques of mutation detection is provided herein.

**Exemplary applications**

The methods and compositions of the present invention can be used with a variety of techniques, both currently in use and to be developed, in which hybridization of an oligonucleotide to another nucleic acid is involved. These include, but are not limited to, techniques in which hybridization of an oligonucleotide to a target nucleic acid is the endpoint; techniques in which hybridization of one or more oligonucleotides to a target nucleic acid precedes one or more polymerase-mediated elongation steps which use the oligonucleotide as a primer and the target nucleic acid as a template; techniques in which hybridization of an oligonucleotide to a target nucleic acid is used to block extension of another primer; techniques in which hybridization of an oligonucleotide to a target nucleic acid is followed by hydrolysis of the oligonucleotide to release an attached label; and techniques in which two or more oligonucleotides are hybridized to a target nucleic acid and interactions between the multiple oligonucleotides are measured. Conditions for hybridization of oligonucleotides, and factors which influence the degree and specificity of
hybridization, such as temperature, ionic strength and solvent composition, are well-known to those of skill in the art. See, for example, Sambrook et al., supra; Ausubel, et al., supra; M.A. Innis et al. (eds.) PCR Protocols, Academic Press, San Diego, 1990; B.D. Hames et al. (eds.) Nucleic Acid Hybridisation: A Practical Approach, IRL Press, Oxford, 1985; and van Ness et al. (1991) Nucleic Acids Res. 19:5143-5151.

Hybridization probes

In one aspect of the present invention, one or more MGB-oligonucleotide conjugates can be used as probe(s) to identify a target nucleic acid by assaying hybridization between the probe(s) and the target nucleic acid. A probe may be labeled with any detectable label, or it may have the capacity to become labeled either before or after hybridization, such as by containing a reactive group capable of association with a label or by being capable of hybridizing to a secondary labeled probe, either before or after hybridization to the target. Conditions for hybridization of nucleic acid probes are well-known to those of skill in the art. See, for example, Sambrook et al., supra; Ausubel et al., supra; Innis et al., supra; Hames et al., supra; and van Ness et al., supra.

Hybridization can be assayed (i.e., hybridized nucleic acids can be identified) by distinguishing hybridized probe from free probe by one of several methods that are well-known to those of skill in the art. These include, but are not limited to, attachment of target nucleic acid to a solid support, either directly or indirectly (by hybridization to a second, support-bound probe or interaction between surface-bound and probe-conjugated ligands) followed by direct or indirect hybridization with probe, and washing to remove unhybridized probe; determination of nuclease resistance; buoyant density determination; affinity methods specific for nucleic acid duplexes (e.g., hydroxyapatite chromatography); interactions between multiple probes hybridized to the same target nucleic acid; etc. See, for example, Falkow et al., U.S. Patent No. 4,358,535; Urdea et al., U.S. Patent Nos. 4,868,105 and 5,124,246; Freifelder, Physical Biochemistry, Second Edition, W. H. Freeman & Co., San Francisco, 1982; Sambrook, et al., supra; Ausubel et al., supra; Hames et al., supra; and other related references. The duplex-stabilizing capability of MGB-oligonucleotide conjugates makes hybridization possible under more stringent conditions, wherein potentially occluding secondary structure in the target nucleic acid can be minimized.
**Amplification primers**

Amplification procedures are those in which many copies of a target nucleic acid sequence are generated, usually in an exponential fashion, by sequential polymerization and/or ligation reactions. Many amplification reactions, such as PCR, utilize reiterative primer-dependent polymerization reactions. A primer is a nucleic acid that is capable of hybridizing to a second, template nucleic acid and that, once hybridized, is capable of being extended by a polymerizing enzyme (in the presence of nucleotide substrates), using the second nucleic acid as a template. Polymerizing enzymes include, but are not limited to, DNA and RNA polymerases and reverse transcriptases, etc. Conditions favorable for polymerization by different polymerizing enzymes are well-known to those of skill in the art. See, for example, Sambrook et al., supra; Ausubel, et al., supra; Innis et al., supra. Generally, in order to be extendible by a polymerizing enzyme, a primer must have an unblocked 3'-end, preferably a free 3' hydroxyl group. The product of an amplification reaction is an extended primer, wherein the primer has been extended by a polymerizing enzyme.

Thus, in one embodiment of the invention, the methods and compositions disclosed and claimed herein are useful in improved amplification reactions such as PCR. See, e.g., U.S. Patents 4,683,202; 4,683,195 and 4,800,159; Mullis and Faloona, supra; and Saiki et al., supra. The polymerization step of PCR is most often catalyzed by a thermostable polymerizing enzyme, such as a DNA polymerase isolated from a thermophilic bacterium, because of the elevated temperatures required for the denaturation step of PCR. As discussed supra, one of the problems heretofore associated with the practice of PCR is the requirement for relatively long oligonucleotide primers, having sufficient hybrid stability to serve as primers at the elevated temperatures under which PCR is conducted.

MGB-oligonucleotide conjugates are useful as primers in amplification reactions such as PCR, since conjugation of a MGB to an oligonucleotide increases hybrid stability, thereby significantly extending the lower limit of useful primer length. In addition, MGB-oligonucleotide conjugates are useful in specialized PCR protocols wherein reduced primer length is desirable. These include, but are not limited to, differential display, in which optimal primer length is below 10 nucleotides, random amplification of polymorphism in DNA (RAPD) techniques, and amplification length polymorphism analyses. Liang et al., supra; Williams et al., supra.
The improvements provided by the present invention are applicable to any type of assay or procedure in which PCR or a related amplification technique is used, including, but not limited to, hydrolyzable probe assays, priming with allele-specific oligonucleotides (ASOs), fragment length polymorphism analysis, single nucleotide polymorphism (SNP) analysis and microsatellite analysis, for example. These and other techniques are useful in gene mapping, in the identification and screening of disease-related genes, and in pharmacogenetics, to name just a few applications.

Assays utilizing labeled probes; including hydrolyzable probe assays

Additional uses for MGB-oligonucleotide conjugates are found in assays in which a labeled probe is hybridized to a target and/or an extension product of a target, and a change in the physical state of the label is effected as a consequence of hybridization. A probe is a nucleic acid molecule that is capable of hybridizing to a target sequence in a second nucleic acid molecule. By way of example, one assay of this type, the hydrolyzable probe assay, takes advantage of the fact that many polymerizing enzymes, such as DNA polymerases, possess intrinsic 5'-3' exonucleolytic activities. Accordingly, if a probe is hybridized to a sequence that can serve as a template for polymerization (for instance, if a probe is hybridized to a region of DNA located between two amplification primers, during the course of an amplification reaction), a polymerizing enzyme that has initiated polymerization at an upstream amplification primer is capable of exonucleolytically digesting the probe. Any label attached to such a probe will be released, if the probe is hybridized to its target and if amplification is occurring across the region to which the probe is hybridized. Released label is separated from labeled probe and detected by methods well-known to those of skill in the art, depending on the nature of the label. For example, radioactively labeled fragments can be separated by thin-layer chromatography and detected by autoradiography; while fluorescently-labeled fragments can be detected by irradiation at the appropriate excitation wavelengths with observation at the appropriate emission wavelengths. See, e.g., U.S. Patent No. 5,210,015.

In a variation of this technique, a probe contains both a fluorescent label and a quenching agent, which quenches the fluorescence emission of the fluorescent label. In this case, the fluorescent label is not detectable until its spatial relationship to the quenching agent has been altered, for example by exonucleolytic release of the fluorescent label from the probe. Thus, prior to hybridization to its target sequence, the dual
fluorophore/quencher labeled probe does not emit fluorescence. Subsequent to hybridization of the fluorophore/quencher-labeled probe to its target, it becomes a substrate for the exonucleolytic activity of a polymerizing enzyme which has initiated polymerization at an upstream primer. Exonucleolytic degradation of the probe releases the fluorescent label from the probe, and hence from the vicinity of the quenching agent, allowing detection of a fluorescent signal upon irradiation at the appropriate excitation wavelengths. This method has the advantage that released label does not have to be separated from intact probe. Multiplex approaches utilize multiple probes, each of which is complementary to a different target sequence and carries a distinguishable label, allowing the assay of several target sequences simultaneously.

The use of MGB-oligonucleotide conjugates in this and related methods allows greater speed, sensitivity and discriminatory power to be applied to these assays. In particular, the enhanced ability of MGB-oligonucleotide conjugates to allow discrimination between a perfect hybrid and a hybrid containing a single-base mismatch will facilitate the use of hydrolyzable probe assays in the identification of single-nucleotide polymorphisms and the like. Examples 2 and 3, infra, provide several examples of the utility of MGB-oligonucleotide conjugates in this type of assay. It will be clear to those of skill in the art that compositions and methods, such as those of the invention, that are capable of discriminating single-nucleotide mismatches will also be capable of discriminating between sequences that have multiple mismatches with respect to one another.

**Fluorescence energy transfer**

In further embodiments of the invention, MGB-oligonucleotide conjugates can be used in various techniques which involve multiple fluorescently-labeled probes. In some of these assays, fluorescence and/or changes in properties of a fluorescent label are used to monitor hybridization. For example, fluorescence resonance energy transfer (FRET) has been used as an indicator of oligonucleotide hybridization. In one embodiment of this technique, two probes are used, each containing a different fluorescent label. One of the labels is a fluorescence donor, and the other is a fluorescence acceptor, wherein the emission wavelengths of the fluorescence donor overlap the absorption wavelengths of the fluorescence acceptor. The sequences of the probes are selected so that they hybridize to adjacent regions of a target nucleic acid, thereby bringing the fluorescence donor and the fluorescence acceptor into close proximity, if target is present. In the presence of target
nucleic acid, irradiation at wavelengths corresponding to the absorption wavelengths of the fluorescence donor will result in emission from the fluorescence acceptor. These types of assays have the advantage that they are homogeneous assays, providing a positive signal without the necessity of removing unreacted probe. For further details and additional examples, see, for example, European Patent Publication 070685; and Cardullo, et al. (1988) Proc. Natl. Acad. Sci. USA 85: 8790-8794. Additional embodiments of the present invention will be found in these and related techniques in which interactions between two different oligonucleotides that are hybridized to the same target nucleic acid are measured. The selection of appropriate fluorescence donor/fluorescence acceptor pairs will be apparent to one of skill in the art, based on the principle that, for a given pair, the emission wavelengths of the fluorescence donor will overlap the absorption wavelengths of the fluorescence acceptor. The enhanced ability of MGB-oligonucleotide conjugates to distinguish perfect hybrids from hybrids containing a single base mismatch facilitates the use of FRET-based techniques in the identification of single-nucleotide polymorphisms and the like.

Use of MGB-oligonucleotide conjugates in assays involving fluorescence quenching

In a further embodiment of the invention, MGB-oligonucleotide conjugates are useful in assays which utilize the principles of fluorescence quenching. In one version of this type of assay, the principles of fluorescence quenching are combined with those of hydrolyzable probes, as discussed supra. In this case, an oligonucleotide probe contains a fluorescent label at one end (usually the 5'-end) and a quenching agent at the opposite end (usually the 3'-end). Exemplary fluorescent labels include, but are not limited to, fluoresceins, cyanines, rhodamines and phycoerythrins. Exemplary quenching agents include, but are not limited to, rhodamines, including tetramethylrhodamine (TAMRA), and compound capable of absorbing UV or visible light. The preferred labels are fluorescein and its derivatives and preferred quenching agents are rhodamine derivatives, particularly TAMRA. When the probe is free in solution or hybridized to its target, irradiation of the fluorophore at the appropriate excitation wavelengths fails to cause fluorescent emission, due to the proximity of the quenching agent to the fluorophore on the oligonucleotide. However, if probe, that has hybridized to its target, is subjected to exonucleolytic hydrolysis by a polymerizing enzyme that has initiated polymerization at an
upstream primer, the fluorophore will be released from the oligonucleotide. Subsequent to its release from the oligonucleotide, the fluorophore will be capable of fluorescing upon excitation at the appropriate wavelengths, since it has been released from the vicinity of the quencher. MGB-oligonucleotide conjugates, by enhancing the ability to distinguish perfect hybrids from hybrids containing a single base mismatch, facilitate the use of oligonucleotides containing fluorophore/quencher combinations in the identification of single-nucleotide polymorphisms and the like. Exemplary oligonucleotides for use in this aspect of the invention contain a conjugated fluorophore, a conjugated quencher and a conjugated MGB. This type of assay is becoming increasingly important, especially in clinical applications, because it is a homogeneous assay (i.e., no product separation steps are required for analysis) in which the results can be monitored in real time. See, for example, Wittwer et al. (1997) BioTechniques 22:130-138. Rapid, fluorescence-based molecular assays find use in, for example, real-time surgical and therapeutic applications, as well.

Additional assays involving the principles of fluorescence quenching will be apparent to those skilled in the art, as will the advantages of using MGB-oligonucleotide conjugates in such assays. It will also be clear to those of skill in the art that fluorescently-labeled MGB-oligonucleotide conjugates provide improvements in speed, sensitivity, specificity and discriminatory power in the practice of all types of hybridization assays.

**PCR clamping**

As described herein, the ability of a MGB-oligonucleotide conjugate (in which the MGB is conjugated to the 5'-end of the oligonucleotide) to block elongation from an upstream primer demonstrates that MGB-oligonucleotide conjugates find use and provide improvements in techniques such as PCR clamping. See, for example, Giovannangeli et al. (1993) Proc. Natl. Acad. Sci. USA 90:10013-10017. Additional modifications of MGB-oligonucleotide conjugates as described *infra*, such as inclusion of phosphorothioate or other modified internucleotide linkages at the 5'-end of the oligonucleotide, will further increase the usefulness of the compositions of the invention in techniques such as PCR clamping.

**Assays involving oligonucleotide ligation**

MGB-oligonucleotide conjugates are useful in assays in which two or more oligonucleotides, complementary to adjacent sites on a target nucleic acid, are hybridized to
adjacent sites on the target nucleic acid and ligated to one another. See, for example, European Patent Publication 320,308; European Patent Publication 336,731; and U.S. Patent No. 4,883,750. Conditions for ligation are well-known to those of skill in the art. See, for example, Sambrook et al., supra; Ausubel, et al., supra; Innis et al., supra.

Ligated nucleic acids can be identified, for example, by an increase in size of the product compared to the starting oligonucleotides. The ability to use shorter oligonucleotides in these types of ligation assay enables smaller, more precise regions of sequence to be probed, which is especially useful in assays based on short regions of homology. Also, as in the case with hybridization assays, use of MGB-oligonucleotide conjugates in ligation assays involving ligation of oligonucleotides allows more efficient discrimination between perfect hybrids and single-base mismatches, which is especially important in oligonucleotide ligation assays. Furthermore, ligation assays often have very narrow temperature optima. The ability of MGB conjugation to raise the Tm of an oligonucleotide allows the temperature optima of ligation assays to be expanded.

cDNA synthesis

The high binding affinity of MGB-oligonucleotide conjugates enables hybridization of shorter oligonucleotides under more stringent conditions. This is important in the synthesis of cDNA from a mRNA template. cDNA synthesis, as commonly practiced, utilizes a reverse transcriptase enzyme to copy a mRNA template into cDNA. The primer for reverse transcription is normally oligodeoxynucleotide, which is complementary to the polyadenylate tail found at the 3' end of most mRNA molecules. Because hybridization between oligodeoxynucleotide and polyadenylate is relatively weak, cDNA synthesis reactions must usually be conducted under conditions of low stringency. However, under such conditions, mRNA molecules are known to readily adopt intramolecular secondary structures, which act as blocks to elongation by reverse transcriptase, leading to production of short, partial cDNA molecules. The increased hybridization strength of MGB-oligonucleotide conjugates allows cDNA synthesis to proceed under more stringent conditions, wherein secondary structure in the mRNA template is minimized, leading to the synthesis of longer cDNA products. Hence, a MGB-oligonucleotide conjugate is used as a primer for cDNA synthesis and is extended by a polymerizing enzyme in the synthesis of a cDNA molecule. As an example, oligodeoxynucleotide conjugated to a MGB is used as a primer for cDNA synthesis. MGB-oligonucleotide conjugates in which the oligonucleotide
sequence is complementary to an internal region of a mRNA template, can also be used for
cDNA synthesis. Similarly, MGB-oligonucleotide conjugates can be used in procedures
such as cDNA indexing, described supra. Accordingly, use of the methods and
compositions of the invention allows longer cDNA molecules to be obtained, compared to
those obtained by the practices of the prior art.

Nucleic Acid Sequencing Systems

In one embodiment of the invention, a collection of all possible n-mer
oligonucleotides (where n is an integer less than about 10) are used in a hydrolyzable probe
assay to determine a nucleotide sequence. Each oligonucleotide is uniquely labeled and
analysis of released label indicates which of the oligonucleotides has hybridized to the
target sequence. Alignment of the sequences of the oligonucleotides which have
hybridized provides the nucleotide sequence.

MGB-oligonucleotide conjugates are also useful in primer-dependent methods of
DNA sequencing, such as the chain-termination method and its derivatives, originally
described by Sanger et al., supra. Use of MGB-oligonucleotide conjugates in chain-
termination sequencing allows the use of shorter primers at higher stringency, and enables a
greater degree of mismatch discrimination during sequencing. Examples include, but are
not limited to, a search for genes sharing a short region of homology (on the order of a few
amino acids) and sequencing in a region in which very little existing sequence information
is available. MGB-oligonucleotide conjugates are useful in such short primer sequencing
techniques.

Oligonucleotide Arrays

In another embodiment of the present invention, MGB-oligonucleotide conjugates
are also useful in procedures which utilize arrays of oligonucleotides, such as sequencing
by hybridization and array-based analysis of gene expression. In these procedures, an
ordered array of oligonucleotides of different known sequences is used as a platform for
hybridization to one or more test polynucleotides, nucleic acids or nucleic acid populations.
Determination of the oligonucleotides which are hybridized and alignment of their known
sequences allows reconstruction of the sequence of the test polynucleotide. See, for
example, U.S. Patent Nos. 5,492,806; 5,525,464; 5,556,752; and PCT Publications WO
92/10588 and WO 96/17957. Materials for construction of arrays include, but are not
limited to, nitrocellulose, glass, silicon wafers, optical fibers and other materials suitable for construction of arrays such as are known to those of skill in the art.

A major problem with current array-based sequencing and analysis methods is that, since the different oligonucleotides in an array will have different base compositions, each will have a different T_m. Hence, it is difficult to determine the stringency conditions that will provide maximum sensitivity, while retaining the ability to distinguish single-base mismatches, which is a particularly important consideration for most, if not all, applications of array technology. Use of MGB-oligonucleotide conjugates in array-based sequencing and analysis techniques provides a solution to this problem, because conjugation of a MGB to an oligonucleotide makes its T_m relatively independent of base composition. Thus, for a population of MGB-oligonucleotide conjugates of a given length, the T_m for a perfect hybrid falls within a relatively narrow temperature range regardless of sequence. At the same time, the T_m for a single nucleotide mismatch is well below the T_m of the perfect match. See Examples 5 and 6, Tables 11 and 12, infra, where data are presented to show that, while differences in T_m related to base composition are minimized for MGB-oligonucleotide conjugates, the conjugates nevertheless retain their discriminatory ability. Thus, arrays designed such that all oligonucleotides are the same length and are present as their MGB conjugates exhibit minimal variation in T_m among the different oligonucleotides in the array, enabling more uniform hybridization conditions for the entire array. A further advantage to the use of MGB-oligonucleotide conjugates in these techniques is that it provides greater sensitivity, by allowing the use of shorter oligonucleotides, at higher temperatures (and hence higher stringency), while retaining single-nucleotide resolution.

An additional application of the present invention to array technology is in the examination of patterns of gene expression in a particular cell or tissue. In this case, oligonucleotides or polynucleotides corresponding to different genes are arrayed on a surface, and a nucleic acid sample from a particular cell or tissue type, for example, is incubated with the array under hybridization conditions. Detection of the sites on the array at which hybridization occurs allows one to determine which oligonucleotides have hybridized, and hence which genes are active in the particular cell or tissue from which the sample was derived.

Array methods can also be used for identification of mutations, where wild-type and mutant sequences are placed in an ordered array on a surface. Hybridization of a
polynucleotide sample to the array under stringent conditions, and determination of which oligonucleotides in the array hybridize to the polynucleotide, allows determination of whether the polynucleotide possesses the wild-type or the mutant sequence. The increased discriminatory abilities of MGB-oligonucleotide conjugates are especially useful in this application of array technology.

Structural considerations

Oligonucleotide, polynucleotide and nucleic acid are used interchangeably to refer to single- or double-stranded polymers of DNA or RNA (or both) including polymers containing modified or non-naturally-occurring nucleotides, or to any other type of polymer capable of stable base-pairing to DNA or RNA including, but not limited to, peptide nucleic acids (Nielsen et al. (1991) Science 254:1497-1500; and Demidov et al. (1995) Proc. Natl. Acad. Sci. USA 92:2637-2641), bicyclo DNA oligomers (Bolli et al. (1996) Nucleic Acids Res. 24:4660-4667) and related structures. One or more MGB moieties and/or one or more labels, quenching agents, etc. can be attached at the 5' end, the 3' end or in an internal portion of the oligonucleotide. Preferred MGB moieties include multimers of 1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate and multimers of N-methylpyrrole-4-carbox-2-amide. Particularly preferred are the trimer of 1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate (CDPI3) and the pentamer of N-methylpyrrole-4-carbox-2-amide (MPC5).

Modified nucleotides including pyrazolopyrimidines (PCT WO 90/14353; and co-owned U.S. Patent Application Serial No. 09/054,830), 7-deazapurines and their derivatives are preferred; additional modified nucleotides are known to those of skill in the art. Oligonucleotides can be obtained from nature or, more preferably, chemically synthesized using techniques that are well-known in the art. See, for example, Caruthers, U.S. Patent No. 4,458,066. Enzymatic synthesis and/or modification of oligonucleotides is also encompassed by the present invention. Preferred in the present invention are DNA oligonucleotides that are single-stranded and have a length of 100 nucleotides or less, more preferably 50 nucleotides or less, still more preferably 30 nucleotides or less and most preferably 20 nucleotides or less.

MGB-oligonucleotide conjugates can comprise one or more modified bases, in addition to the naturally-occurring bases adenine, cytosine, guanine, thymine and uracil.
Modified bases are considered to be those that differ from the naturally-occurring bases by addition or deletion of one or more functional groups, differences in the heterocyclic ring structure (i.e., substitution of carbon for a heteroatom, or vice versa), and/or attachment of one or more linker arm structures to the base. All tautomeric forms of naturally-occurring bases, modified bases and base analogues are useful in the MGB-oligonucleotide conjugates of the invention.

Similarly, modified sugars or sugar analogues can be present in one or more of the nucleotide subunits of a MGB-oligonucleotide conjugate. Sugar modifications include, but are not limited to, attachment of substituents to the 2', 3' and/or 4' carbon atom of the sugar, different epimeric forms of the sugar, differences in the α- or β- configuration of the glycosidic bond, anomeric changes, etc. Sugar moieties include, but are not limited to, pentose, deoxypentose, hexose, deoxyhexose, ribose, deoxyribose, glucose, arabinose, pentofuranose, xylose, lyxose, and cyclopentyl.

Modified internucleotide linkages can also be present in MGB-oligonucleotide conjugates. Such modified linkages include, but are not limited to, peptide, phosphate, phosphodiester, phosphotriester, alkylphosphate, alkanephosphonate, thiophosphate, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, substituted phosphoramidate and the like. Various modifications of bases, sugars and/or internucleotide linkages, that are compatible with their use in oligonucleotides serving as probes and/or primers, will be apparent to those of skill in the art.

Modified bases for use in the present invention also include, but are not limited to, pyrazolo[3,4-d]pyrimidine analogues of adenine and guanine, as disclosed in co-owned PCT Publication WO 90/14353. Preferred base analogues of this type include the guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (ppG) and the adenine analogue 4-amino-1H-pyrazolo[3,4-d]pyrimidine (ppA). Also of use is the xanthine analogue 1H-pyrazolo[3,4-d]pyrimidin-4(5H)-6(7H)-dione (ppX). These base analogues, when present in an oligonucleotide, strengthen hybridization and improve mismatch discrimination. See co-owned, copending U.S. Patent Application Serial No. 09/054,830. Example 3, infra, shows the beneficial effect of including a pyrazolo[3,4-d]pyrimidine base analogue in a MGB-oligonucleotide conjugate used in a hydrolyzable probe assay (Figures 4 and 5). Modified bases for use in the present invention can also include those for use in selective binding complementary oligonucleotides, as disclosed in co-owned PCT
Publication WO97/12896. Particularly preferred are those which modify the minor groove, such as 2-amino adenine and 2-thiothymine.

MGB-oligonucleotide conjugates can contain other pendant moieties, in addition to the MGB. Examples include, but are not limited to, detectable labels (see elsewhere in the present specification); crosslinking agents such as those disclosed in U.S. Patent No. 5,659,022 and PCT Publications WO 90/14353, WO93/03736, WO94/17092, and WO96/40711; tail moieties such as those disclosed in U.S. Patents 5,419,966 and 5,512,667; peptide linkers such as those disclosed in U.S. Patent No. 5,574,142; sterols and other lipophilic groups such as those disclosed in U.S. Patent No. 5,646,126; intercalating agents, reporter groups, electrophilic groups and chelating agents, such as are known to those of skill in the art, and other pendant moieties known to those of skill in the art.

The MGB can be attached at either or both ends of the oligonucleotide. In addition or alternatively, one or more MGBs can be attached in the interior of the oligonucleotide, depending on the length of the oligonucleotide. In general, conjugation of a MGB to either end of an oligonucleotide would provide the greatest degree of hybrid stability, since melting of an oligonucleotide duplex begins at the termini. Nonetheless, if both ends of a duplex formed by an oligonucleotide are relatively stable, for example, due to a high G+C content, attachment of a MGB in the interior of an oligonucleotide (for instance, near an A+T-rich sequence) could also enhance stability. The intended use of the MGB-oligonucleotide conjugate may also place limitations on the location of the conjugated MGB. For instance, if an oligonucleotide is designed to be used as a primer, the 3′-hydroxy group must be free and capable of being elongated by a polymerizing enzyme. Alternatively, an assay that requires an oligonucleotide possessing a labeled 5′-end would require internal or 3′-end attachment of a MGB.

The location of a MGB within a MGB-oligonucleotide conjugate might also affect the discriminatory properties of such a conjugate. An unpaired region within a duplex will result in changes in the shape of the minor groove in the vicinity of the mispaired base(s). Since MGBs fit best within the minor groove of a perfectly-matched DNA duplex, mismatches resulting in shape changes in the minor groove would reduce binding strength of a MGB to a region containing a mismatch. Hence, the ability of a MGB to stabilize such a hybrid would be decreased, thereby increasing the ability of a MGB-oligonucleotide conjugate to discriminate a mismatch from a perfectly-matched duplex. On the other hand,
if a mismatch lies outside of the region complementary to a MGB-oligonucleotide conjugate, discriminatory ability for unconjugated and MGB-conjugated oligonucleotides of equal length is expected to be approximately the same. Since the ability of an oligonucleotide probe to discriminate single base pair mismatches depends on its length, shorter oligonucleotides are more effective in discriminating mismatches. The primary advantage of the use of MGB-oligonucleotides conjugates in this context lies in the fact that much shorter oligonucleotides compared to those used in the prior art (i.e., 20-mers or shorter), having greater discriminatory powers, can be used, due to the pronounced stabilizing effect of MGB conjugation.

It has also been discovered that substitution of inosine for guanosine in a MGB-oligonucleotide conjugate can enhance hybrid stability. Without wishing to be bound by any particular theory, it is likely that inosine substitution makes the local shape of the minor groove more favorable for interaction with a MGB, thereby increasing the strength of the MGB-minor groove interaction. This contribution to duplex stability offsets the weaker base-pairing of the I:C base pair compared to the G:C base pair. Example 4 provides data showing increased Tm's for hybrids in which one of the strands is an inosine-containing oligonucleotide.

It will be apparent to those of skill in the art that additional minor groove binding moieties, related to those disclosed herein, will function similarly to facilitate hybridization and primer function of oligonucleotides.

Labels

MGB-oligonucleotide conjugates can be labeled with any label known in the art of nucleic acid chemistry. Detectable labels or tags suitable for use with nucleic acid probes are well-known to those of skill in the art and include, but are not limited to, radioactive isotopes, chromophores, fluorophores, chemiluminescent and electrochemiluminescent agents, magnetic labels, immunologic labels, ligands and enzymatic labels. Suitable labels further include mass labels and those used in deconvolution of combinatorial chemistry libraries, for example, tags that can be recognized by high performance liquid chromatography (HPLC), gas chromatography, mass spectrometry, etc.

Methods for probe labeling are well-known to those of skill in the art and include, for example, chemical and enzymatic methods. By way of example, methods for
incorporation of reactive chemical groups into oligonucleotides, at specific sites, are well-known to those of skill in the art. Oligonucleotides containing a reactive chemical group, located at a specific site, can be combined with a label attached to a complementary reactive group (e.g., an oligonucleotide containing a nucleophilic reactive group can be reacted with a label attached to an electrophilic reactive group) to couple a label to a probe by chemical techniques. Exemplary labels and methods for attachment of a label to an oligonucleotide are described, for example, in U.S. Patent No. 5,210,015; Kessler (ed.), *Nonradioactive Labeling and Detection of Biomolecules*, Springer-Verlag, Berlin, 1992; Kricka (ed.) *Nonisotopic DNA Probe Techniques*, Academic Press, San Diego, 1992; Howard (ed.) *Methods in Nonradioactive Detection*, Appleton & Lange, Norwalk, 1993.

Non-specific chemical labeling of an oligonucleotide can be achieved by combining the oligonucleotide with a chemical that reacts, for example, with a particular functional group of a nucleotide base, and simultaneously or subsequently reacting the oligonucleotide with a label. See, for example, Draper *et al.* (1980) *Biochemistry* 19:1774-1781. Enzymatic incorporation of label into an oligonucleotide can be achieved by conducting enzymatic modification or polymerization of an oligonucleotide using labeled precursors, or by enzymatically adding label to an already-existing oligonucleotide. See, for example, U.S. Patent No. 5,449,767. Examples of modifying enzymes include, but are not limited to, DNA polymerases, reverse transcriptases, RNA polymerases, *etc.* Examples of enzymes which are able to add label to an already-existing oligonucleotide include, but are not limited to, kinases, terminal transferases, ligases, glycosylases, *etc.*

In certain embodiments of the present invention, MGB-oligonucleotide conjugates comprising fluorescent labels (fluorophores) and/or fluorescence quenching agents are used. In a preferred embodiment, a MGB-oligonucleotide conjugate contains both a fluorophore and a quenching agent. Fluorescent labels include, but are not limited to, fluoresceins, rhodamines, cyanines, phycoerythrins, and other fluorophores as are known to those of skill in the art. Quenching agents are those substances capable of absorbing energy emitted by a fluorophore so as to reduce the amount of fluorescence emitted (*i.e.*, quench the emission of the fluorescent label). Different fluorophores are quenched by different quenching agents. In general, the spectral properties of a particular fluorophore/quenching agent pair are such that one or more absorption wavelengths of the quencher overlaps one or more of the emission wavelengths of the fluorophore.
preferred fluorophore/quencher pair is fluorescein/tetramethylrhodamine; additional fluorophore/quencher pair can be selected by those of skill in the art by comparison of emission and excitation wavelengths according to the properties set forth above.

For use in an amplification assay which involves elevated temperatures, such as PCR, or other procedures utilizing thermostable enzymes, the label will be stable at elevated temperatures. For assays involving polymerization, the label will be such that it does not interfere with the activity of the polymerizing enzyme. Label can be present at the 5' and/or 3' end of the oligonucleotide, and/or may also be present internally. The label can be attached to any of the base, sugar or phosphate moieties of the oligonucleotide, or to any linking group that is itself attached to one of these moieties.

EXAMPLES

Example 1: MGB-oligonucleotide conjugates as PCR primers

In this example, we show that a modification which greatly improves hybrid stability of a short oligonucleotide also allows the oligonucleotide to serve as a PCR primer. CDPI₃, the trimer of 1,2-dihydro-(3H)-pyrrolo[3,2-c]indole-7-carboxylate, or CDPI₁; is a synthetic non-reactive derivative of a subunit of the antitumor antibiotic CC-1065 (Hurley et al. (1984) Science 226:843-844). This oligopeptide is a DNA minor groove binder (MGB), with a very high affinity for the minor groove of A-T-rich double-stranded DNA. It has previously been reported that, when compared to unmodified oligonucleotides of the same length, CDPI₃-oligonucleotide conjugates form unusually stable and specific hybrids with complementary single-stranded DNA (Lukhtanov et al. (1995) Bioconjugate Chem. 6:418-426; Afonina et al. (1996) Proc. Natl. Acad. Sci. USA 93:3199-3204). This example demonstrates that conjugates of short oligonucleotides with CDPI₃ make effective primers for PCR, thus improving the yield and accuracy of priming with short primers. Oligonucleotides as short as 8-mers and G-C-rich 6-mers are able to specifically prime the amplification reaction when conjugated to a MGB. Thus, conjugation of a MGB to an oligonucleotide under the conditions described herein does not interfere with the ability of the oligonucleotide 3'-end to be extended by a polymerizing enzyme.
Oligonucleotides and oligonucleotide conjugates

Oligonucleotides used in this study were complementary to various regions of the M13mp19 genome. Oligonucleotide synthesis was performed on an Applied Biosystems Model 394 DNA synthesizer using the 1 µmol coupling program supplied by the manufacturer. CDPI₃ was postsynthetically conjugated to the 5'-end of ODNs as described (Lukhtanov et al., supra). ODNs were purified by HPLC on a reverse-phase column eluted by an acetonitrile gradient (usually 0-45%) in 100 mM triethylamine acetate (pH 7.5) buffer. Purity of unmodified ODNs was evaluated by electrophoresis on an 8% polyacrylamide-8 M urea gel with subsequent visualization by silver staining (Daichi). Purity of the oligonucleotide-CDPI₃ conjugates was verified by analytical HPLC as described above. All oligonucleotide preparations were >95% pure.

The preparation of CDPI₃ is described in WO 96/32496 (Reaction scheme 1 and accompanying text) and formation of the MGB-oligonucleotide conjugate was accomplished by reaction of the 2,3,5,6-tetrafluorophenyl ester of CDPI₃ with an oligonucleotide with a 5'-aminohexyl phosphate ester (see Reaction Scheme 3 in WO 96/32496). Figure 1 depicts the CDPI₃ molecule and the structures of the linkers through which it is attached to the 5' or 3' ends of an oligonucleotide. The sequences of the oligonucleotides used in this example are shown in Table 1.

Thermal denaturation studies

Hybrids formed between MGB-oligonucleotide conjugates or unmodified oligonucleotides and their complements were melted at a rate of 0.5°C/min in 140 mM KCl, 10 mM MgCl₂ and 20 mM HEPES-HCl (pH 7.2) on a Lambda 2S (Perkin Elmer) spectrophotometer with a PTP-6 automatic multicell temperature programmer. Each oligonucleotide (2 µM) was mixed with sufficient complementary oligonucleotide to give a 1:1 ratio. Prior to melting, samples were denatured at 100°C and then cooled to the starting temperature over a 10 min period. The melting temperatures (Tₘ) of the hybrids were determined from the derivative maxima and collected in Table 1. In the “sequence” column of Table 1, MGB refers to the presence of CDPI₃ conjugated through a hexylamine linker esterified to the 5'-phosphate group of the oligonucleotide (see Figure 1).
<table>
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<tr>
<th>ODN</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Length (ntds)</th>
<th>% GC</th>
<th>Sequence</th>
<th>Location on M13mp19 genome</th>
<th>SEQ ID NO.</th>
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<td>1</td>
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<td>16</td>
<td>37.5</td>
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<td>2</td>
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<td>12</td>
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<td>5'-ATAAAAACAGAGG-3'</td>
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<tr>
<td>2-C</td>
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<td>12</td>
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<td>3</td>
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<tr>
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<td>5'-MGB-TATTTTAGATAACCTT-3'</td>
<td>4756-4771</td>
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</table>
PCR Reactions

All PCR reactions were performed on a Perkin-Elmer Cetus DNA Thermocycler and included: PCR buffer (Promega) with no magnesium, 1.6 mM MgCl₂, 50 μM dNTP, 50 nM each primer, 0.2 μg M13mpl9 DNA and 1-2 Units Taq DNA polymerase (Promega). Final volume for each reaction was 50 μl. The standard PCR profile was as follows: 3 min at 94°C, 30 cycles of 1 min at 94°C, 1.5 min at annealing temperature and 30 s at 72°C, finally followed by 5 min at 72°C and a 4°C soak. For the 8-mers and 6-mers, PCR was performed in the touch-down manner (Don, R.H. et al. (1991) Nucleic Acids Res. 19: 4008) with a starting annealing temperature of 55°C for 8-mer primers and 50°C for 6-mers. Each subsequent cycle had an annealing temperature 1°C lower until 41°C (for 8-mers) or 37°C (for 6-mers) was reached, with the final 15 cycles annealed at these final temperatures. Touch-down PCR has been shown to maximize the yield of product when using short primers (Don, R.H. et al., supra). Amplifications with 16-mer, 12-mer and 10-mer primers were analyzed by electrophoresis of 10 μl of the reaction mixture on a 2% agarose gel and detection of the bands by ethidium bromide staining. Amplifications with 8-mer and 6-mer primers were analyzed by electrophoresis of 5 μl of reaction mixture on an 8% polyacrylamide sequencing gel and detection of the bands by silver staining (Daiichi).

Results

Hybrid Stability

Table 1 presents the melting temperatures (Tₘ) of duplexes formed by the MGB-ODN conjugates with complementary ODNs, showing the effect of a terminally-conjugated CDPI₃ group on duplex stability for duplexes of different lengths and G-C compositions. For the 16-mer duplexes, the largest increase in Tₘ attributable to a tethered MGB (22°C), was obtained when that group was flanked by
a run of seven A-T base pairs (compare oligonucleotides 13-C and 13). A-T-rich sequences of this length form minor grooves which act as good binding sites for the CDPI3 tripeptide. Of all the hybrids examined, the 16-mer duplex formed by oligonucleotide 4-C gave the highest absolute \( T_m \) (66°C). This unusually high \( T_m \) reflects an otherwise G-C-rich duplex which contains six A-T base pairs adjacent to the tethered CDPI3 group. Conversely, a 16-mer duplex with a G-C-rich sequence flanking the MGB conjugation site (12-C) was only 9°C more stable than the unmodified duplex (12). The CDPI3 group in this duplex binds in a less favorable G-C-rich minor groove.

The \( T_m \)s reported for the shorter primers in Table 1 follow the same trends as for the 16-mers. The primers with the A-T-rich regions adjacent to the MGB at the 5'-end had higher \( T_m \)s than those with G-C-rich regions, and they showed a greater increase in \( T_m \) compared to their non-conjugated counterparts. The 10-mers 3-C and 7-C, for instance, had \( T_m \) values of 46-49°C, well within a range adequate for specific PCR priming.

**Priming Ability**

The ODNs and MGB-ODN conjugates were tested as PCR primers using M13mp19 single-stranded DNA as the amplification substrate. Typically, unmodified and CDPI3-conjugated versions of the same oligonucleotide were compared. These were tested in parallel, as reverse primers, using a PCR profile in which only the annealing temperature was varied. A lower than usual concentration of primers (<0.1 \( \mu \)M) was employed when using the MGB-oligonucleotide conjugates. This minimized any spurious interaction of these conjugates with A-T-rich sequences due to the anchor effect of the CDPI3 group (Afonina, I. *et al.*, *supra*). To confirm the specificity of primer binding, the primers were designed such that every amplified product in this study contained a *DdeI* restriction site. In addition to measuring the size of the amplification product (Figures 2 and 3), aliquots of selected
PCR reaction mixtures were treated with *DdeI* and analyzed in a 2% agarose gel. In all cases the expected restriction fragments were obtained.

Figures 2A and 2B demonstrate the improved priming performance of 16-mer MGB-ODN conjugates in comparison with unmodified primers. Conditions of amplification were the same with the exception of annealing temperature, which was 45°C for Figure 2A and 68°C for Figure 2B. While all of these 16-mer oligonucleotides primed at the lower temperature (Figure 2A, see legend for predicted product sizes), only those with a 5'-CDPI₃ group primed at the higher temperature (Figure 2B, see legend for predicted product sizes).

Figure 2C confirms the advantages of conjugation of CDPI₃ to short primers 10 or 12 nucleotides long. Both conjugated primers efficiently amplified the expected sequence (see Figure 2C legend for predicted product sizes). The same primers without a tethered MGB did not generate product detectable by ethidium bromide staining when amplified under the same conditions.

Figure 3 demonstrates that specific priming is possible even for primers as short as an 8-mer (Fig. 4A) and a 6-mer (Fig. 4B). A 10-mer forward primer was used in these reactions. The low levels of product necessitated the use of touch-down PCR (Don, *et al.*, *supra*) and detection of bands by silver staining. In each case a band of expected size (see legends to Figures 3A and 3B for predicted product sizes) was observed only when the reverse primer was conjugated to a CDPI₃ group.

Without wishing to be bound by any particular theory, it appears that the increase in stability of a hybrid comprising a MGB-oligonucleotide conjugate compared to one containing a non-conjugated oligonucleotide is likely to be due to the binding of the tethered MGB in the duplex region. The binding region of the MGB probably spans up to 6 base pairs. Importantly, the 3'-terminus of the MGB-conjugated oligonucleotide is still recognized by the polymerizing enzyme, as primer extension seems to depend only on hybrid stability and is not inhibited by the presence of the MGB.
Example 2: Use of MGB-oligonucleotide conjugates in a hydrolyzable probe assay

In this example, we show that conjugation of MGBs to short oligonucleotides results in improved hybrid stability and improved discrimination between a perfect hybrid and a single-base mismatch, when MGB-short oligonucleotide conjugates are used in a hydrolyzable probe assay. The procedure described by Wittwer et al. (1997a) *BioTechniques* **22:**130-138, was used. In this method, MGB-oligonucleotide conjugates, additionally comprising a fluorophore and a quenching agent, were used as probes in a hydrolyzable probe assay. This type of probe is designed to be complementary to a predicted amplification product, and emits very little or no fluorescence, due to the proximity of the fluorophore to the quenching agent. Formation of a hybrid between the probe and the amplification product produces a structure that is a substrate for exonucleolytic hydrolysis of the probe by a polymerase possessing duplex-specific exonuclease activity, if the polymerase has initiated polymerization at an upstream primer. The exonuclease action will release the fluorophore from the hybridized oligonucleotide and hence from the proximity of the quenching agent, resulting in an increase in fluorescence. Thus, in this assay, increase in fluorescence is dependent upon duplex formation between the fluorophore/quencher-labeled MGB-oligonucleotide conjugate probe and the desired amplification product. See U.S. Patent No. 5,210,015; Livak *et al.* (1995) *PCR Meth. App.* **4:**357-362; and Heid *et al.* (1996) *Genome Res.* **6:**986-994 for further details.
Synthesis of MGB-oligonucleotide conjugates containing fluorophore(s) and quencher(s)

CDP1-CPG supports (Scheme 1)

4-[(2-Phenyl)-1,3-dioxolan-4-yl]-1-butanol (1). To a solution of 1,2,6-trihydroxyhexane (10.0 g, 74.6 mmol) and benzaldehyde dimethylacetal (15.0 g, 98.7 mmol) in dry DMF (10 mL) was added Amberlyst 15 (5.0 g). The mixture was stirred at 100°C for 5 min, then cooled and filtered. The filtrate was concentrated and residue obtained was re-dissolved in ethyl acetate. After being washed with water and brine, the solution was dried over Na₂SO₄. The crude product obtained after evaporation of the solvent was chromatographed on silica eluting with ethyl acetate. Concentration of the proper fractions afforded 11.0 g (66%) of the title product (a mixture of diastereomers) as a colorless oil: ¹H NMR (CDCl₃, 300 MHz, ppm) 7.48 (m, 2H), 7.39 (m, 3H), 5.92 (s, 0.4H), 5.80 (s, 0.6H), 4.3-4.1 (m, 2H), 3.64 (m, 3H), 1.8-1.4 (m, 6H).

2-Phenyl-4-(4-phtalimidobut-1-yl)-1,3-dioxolane (2). A solution of diethyl azodicarboxylate (8.6 g, 49.4 mmol) in 40 mL of THF was slowly added to a cold (ice bath) solution of 1 (10.0 g, 45.0 mmol), triphenylphosphine (13.0 g, 49.5 mmol) and phthalimide (7.3 g, 49.7 mmol) in 60 mL of THF. After being kept at ambient temperature overnight, the solution was concentrated and the residue was triturated with ether (~150 mL) to precipitate triphenylphosphine oxide which was then removed by filtration. The filtrate was concentrated to give an oily residue which was chromatographed on silica eluting with 33% ethyl acetate in hexane. Concentration of the pure product fractions followed by drying under vacuum afforded 14.6 g (92%) of the desired product as a semi-solid (a mixture of diastereomers): ¹H NMR (CDCl₃, 300 MHz, ppm) 7.85 (m, 2H), 7.71 (m, 2H), 7.46 (m, 2H), 7.36 (m, 3H), 5.90 (s, 0.4H), 5.78 (s, 0.6H), 4.3-4.0 (m, 2H), 3.68 (m, 3H), 1.9-1.3 (m, 6H).
6-[(9-Fluorenylethoxycarbonyl)amino]-(R,S)-1,2-hexanediol (3). A suspension of 2 (13.5 g, 38.5 mmol) in 50 mL of ethanol was treated with 1.9 mL (39.1 mmol) of hydrazine monohydrate and heated at reflux for 3 h. The mixture was cooled and acidified with 1M HCl to pH 3. The precipitate formed was filtered off, the filtrate was extracted with ether and concentrated to give a semi-solid. It was suspended in 2-propanol to separate insoluble inorganic salts. After filtration, the solution was concentrated in vacuo to afford 5.0 g of a syrup containing mostly the desired product contaminated with 2-propanol. This material was used in the next reaction without additional purification.

To a solution of the above crude aminodiol in a mixture of methanol (25 mL) and CH₂Cl₂ (10 mL) was added triethylamine (7 mL) followed by 9-fluorenylethyl N-succinimidy carbonate (8.0 g, 23.7 mmol). After being stirred for 1 h, the reaction mixture was treated with acetic acid (5 mL) to neutralize excess triethylamine, and concentrated. Trituration of the residue with water gave a white solid which was washed with water and ether. Drying in vacuo afforded 5.5 g (40%) of analytically pure product: ¹H NMR (DMSO-d₆, 300 MHz, ppm) 7.88 (d, 2H), 7.68 (d, 2H), 7.41 (t, 2H), 7.33 (t, 2H), 4.45 (t, 1H), 4.37 (d, 1H), 4.28 (d, 2H), 4.21 (m, 1H), 3.35 (m, 1H), 3.24 (m, 2H), 2.96 (m, 2H), 1.39 (m, 4H), 1.21 (m, 2H).

6-[(9-Fluorenylethoxycarbonyl)amino]-2-O-(4,4’dimethoxytriphenylmethyl)-(R,S)-1,2-hexanediol (4). To a solution of 3 (2.0 g, 5.6 mmol) in 20 mL of dry pyridine was added N,N-dimethylaminopyridine (0.1 g) and 4,4’dimethoxytrityl chloride (4.0 g, 11.8 mmol). After being stirred for 2 h, the solution was concentrated and the resultant oily residue was re-dissolved in ethyl acetate. The solution was washed with water, brine and dried over Na₂SO₄. Evaporation of the solvent gave crude product which was chromatographed on silica eluting with 50% ethyl acetate in hexane. The title product was obtained as a pale yellow amorphous solid (2.8 g, 79%) after evaporation of the solvent: (CDCl₃, 300
MHz, ppm) 7.77 (d, 2H), 7.59 (d, 2H), 7.5-7.1 (m, 17H), 6.84 (t, 4H), 4.80 (t, 1H), 4.40 (d, 2H), 4.22 (t, 1H), 3.80 (s, 6H), 3.2-3.0 (m, 4H), 1.6-1.1 (m, 6H).

2,3,5,6-Tetrafluorophenyl 6-[N-(9-fluorenylemethoxycarbonyl)amino]-
(R,S)-2-(4,4′-dimethoxytriphenylmethoxy)-hex-1-yl butanedioate (5). To a solution of 4 (1.0 g, 1.6 mmol) in 5 mL of dry pyridine was added succinic anhydride (1.0 g, 10 mmol) followed by 1-methylimidazole (0.02 mL). The reaction mixture was stirred for 8 h at 55°C and treated with water (1 mL). Concentration under vacuum gave an oil which was partitioned between CHCl₃ and cold 10% citric acid. The organic phase was washed with water and dried over Na₂SO₄. Evaporation of the solvent afforded crude acid as an amorphous solid (0.99 g), this material was taken to the next step without further purification.

To a solution of the above acid in 5 mL of dry CH₂Cl₂ was added triethylamine (0.25 mL) followed by 0.25 mL (1.4 mmol) 2,3,5,6-tetrafluorophenyl trifluoroacetate. Gamper, H.B. et al. (1993) *Nucleic Acids Res.* **21**: 145. After being kept at ambient temperature for 15 min, the solution was applied onto a silica gel column. Elution of the column with 33% ethyl acetate in hexane and concentration of the pure product fractions afforded 1.0 g (71%) of the desired TFP ester as a white, amorphous solid: (CDCl₃, 300 MHz, ppm) 7.76 (d, 2H), 7.58 (d, 2H), 7.5-7.1 (m, 17H), 7.00 (m, 1H), 6.84 (t, 4H), 5.1 (m, 1H), 4.79 (t, 1H), 4.38 (d, 2H), 4.22 (t, 1H), 3.78 (s, 6H), 3.2-2.9 (m, 6H), 2.81 (m, 2H), 1.61 (m, 2H), 1.45 (m, 2H), 1.26 (m, 2H).

Preparation of CPG 6. To a solution of 5 (0.5 g, 0.57 mmol) in 20 mL of dry pyridine was added long chain aminoalkyl CPG (500 A) (5.0 g) followed by 1-methylimidazole (1.0 mL). After being swirled for 15 h at ambient temperature, the suspension was treated with acetic anhydride (3 mL) to cap unreacted amino groups (15 min). The CPG was then washed with DMF, acetone, ether and dried. The CPG
was analyzed for DMTr content (Atkinson, T. et al. (1984) Solid-Phase Synthesis of Oligodeoxyribonucleotides by Phosphite-Triester Method. In “Oligonucleotide Synthesis, A practical Approach”. (M. J. Gait, Ed.) pp. 35-81. IRL Press, Washington, DC.) and found to have a loading of 49 μmol/g.

Preparation of CPG 7. CPG 6 (2.8 g) was deprotected by a treatment with 20 mL of 0.2 M 1,8-diazabicyclo[5.4.0]undec-7-ene(1,5-5) (DBU) for 20 min. The CPG was extensively washed with DMF and ether, dried and re-suspended in a solution of CDPI₃-TFP (Lukhtanov, E.A. et al. (1995) Bioconjugate Chem., 6: 418) (200 mg) and N,N-diisopropylethylamine (1 mL) in 8 mL of DMF. After being swirled for 3 days, the CPG was washed with N,N-dimethylacetamide, acetone, ether and dried. Unreacted amino groups were capped by treatment with 10% acetic anhydride in pyridine for 15 min, and the CPG was washed and dried as described above.
Scheme 1: Preparation of CDPI₃-CPG support (below)

Oligonucleotide synthesis using the CDPI₃-CPG support (Scheme 2).

Trityl-off 3'-CDPI₃ oligonucleotides were prepared in 1 µmol scale using standard 3'-phosphoramidite chemistry on the CDPI₃-CPG support (~20-50 mg) on an ABI 394 according to the protocol supplied by the manufacturer with one exception: 0.01 M (instead of the standard 0.1 M) iodine solution in was utilized in the oxidation step to avoid iodination of the CDPI₃ moiety. In order to introduce an amino-linker for the postsynthetic incorporation of the TAMRA dye (see below), protected aminopropyl
ppG and aminopropyl ppA phosphoramidites were utilized at the desired step instead of the standard guanosine or adenosine phosphoramidites, respectively. See co-owned, PCT WO 90/14353.

**Scheme 2:** Introduction of 3’-CDPI₃ residue using the CDPI₃-CPG support (below)

![Diagram of CDPI₃-CPG synthesis and deprotection](image)

**Incorporation of 6-FAM and TET fluorophores (Scheme 3) and isolation of the conjugates.**

6-FAM (6-carboxyfluorescein) or TET (6-carboxy-4,7,2’,7’-tetrachlorofluorescein) was introduced at the last step of the above-described automated oligonucleotide synthesis using corresponding 6-FAM and TET phosphoramidites (Glen Research) according to the protocol supplied by the manufacturer. After deprotection the modified ODNs were purified by reverse-phase
chromatography on a 4.6x250 mm, C-18 column (Dynamax-300, Rainin) eluting with a gradient of acetonitrile (0-60%) buffered at pH 7.5 (0.1 M triethylammonium acetate). The desired fraction was concentrated to a volume of ~50 μL by extraction with n-butanol and then diluted with 2% solution of LiClO₄ in acetone (1.2 mL). The resultant precipitate was collected by centrifugation, the pellet was washed with acetone (2x 1.2 mL) and dried under vacuum.

**Scheme 3**: Introduction of fluorophores at the 5'-end using 6-FAM and TET phosphoramidates (below)

```
R=H 6-FAM (6-carboxy fluorescein)
Glen Research

R=Cl TET (6-carboxy, 4, 7, 2, 7'-tetrachlorofluorescein)
Glen Research
```
Postsynthesis introduction of TAMRA residue (Scheme 4). TAMRA (tetramethylrhodamine) was incorporated into the above conjugates by reaction of the oligonucleotide with TAMRA-N-hydroxysuccinimide ester (Glen Research) according to the protocol supplied by the manufacturer. Under these conditions, the TAMRA moiety is added to an amino group linked to a ppG or ppA residue in the oligonucleotide. Purification of the double dye (fluorescein and TAMRA)-labeled CDPI3-ODN conjugates was accomplished by denaturing 20% PAGE, the desired band was cut out and the conjugate extracted by incubation of the gel slice in 0.1 M triethylammonium acetate (10 mL) (pH 7.5) overnight at 37°C. Finally, the conjugates were isolated from the extract by reverse phase HPLC as described above.
Scheme 4: Postsynthesis introduction of TAMRA quencher at the internal ppG or ppA residues (below)
Assay

Reaction mixtures contained 40 mM NaCl, 20 mM Tris-Cl pH 8.9, 5 mM MgSO₄, 0.05% (w/v) bovine serum albumin, 125 μM each of the four dNTPs, 1 ng template 0.5 μM each primer, 0.5 μM probe (fluorophore/quencher labeled MGB-oligonucleotide conjugate) and 0.5 μl of Taq Polymerase per 10 μl reaction volume. Forty cycles of amplification were conducted. Each cycle was 0 sec at 94°C for denaturation (i.e., temperature was raised to 94°C and immediately lowered to the annealing/elongation temperature), followed by 15 sec at a combined annealing/elongation temperature. The annealing/elongation temperature varied among different reactions and is specified for each particular case.

Assays were conducted following the method of Wittwer et al. (1997a) BioTechniques 22:130-138, and fluorescence measurements were made using a Light Cycler™, available from Idaho Technology. Wittwer et al. (1997b) BioTechniques 22:176-181. Fluorescein fluorescence was determined by excitation at 485 nm and detection of emission between 518-530 nm.

Results

Experiments using different length probes, either unconjugated or conjugated to a MGB (CDPI₃) were conducted at various annealing/elongation temperatures. All probes contained a molecule of carboxamidofluorescein conjugated to the 5'-end via a hexyl linker and a molecule of tetramethylrhodamine (TAMRA) conjugated at the 3'-end, as described supra.

The effect of MGB conjugation on hybridization between perfectly-matched sequences and sequences containing a single nucleotide mismatch was assessed for 12-mer oligonucleotides in a hydrolyzable probe assay. In each experiment, there were four samples, each containing a different probe. The probes were either fully complementary to the target sequence (i.e., a perfect match) or had a single base mismatch, and either contained or lacked a MGB conjugated at the 3'-end of the
oligonucleotide as described *infra*. The experiment was conducted at an annealing/elongation temperature of 65°C. Oligonucleotides which did not contain a MGB gave baseline levels of fluorescence through 30 cycles of amplification. A MGB-conjugated 12-mer with perfect complementarity to target showed gradually increasing fluorescence from the start of the amplification process, with a significant increase in fluorescence beginning at about the 18th cycle. A MGB-conjugated 12-mer with a single-nucleotide mismatch showed fluorescence levels that were close to baseline, and that were clearly distinguishable from the levels generated by the perfectly matched sequence.

The behavior of 10-mer oligonucleotides with and without a conjugated MGB was also examined at an annealing/elongation temperature of 65°C. With 10-mers, the background was higher and more variable. Nevertheless, while only background signal was obtained when an unconjugated 10-mer was used in the assay (with either a perfect match or a single-nucleotide mismatch), signal obtained using a MGB-conjugated 10-mer with perfect complementarity was readily distinguished from that obtained using a MGB-conjugated 10-mer with a single-base mismatch.

From these results it is clear that conjugation of a MGB to a short oligonucleotide greatly stabilizes the hybrids formed by such conjugated oligonucleotides, compared to oligonucleotides not containing an attached MGB. It should also be noted that, in all cases, the difference in hybrid stability (as evidenced by differences in fluorescence levels at later amplification cycles) between fully complementary probes and probes with a single base mismatch is more pronounced for probes with a conjugated MGB (compared to unconjugated probes), showing that conjugation of a MGB helps to increase the discriminatory power of a short oligonucleotide probe.
Example 3: Effect of nucleotide analogues on hybridization strength and discriminatory ability of MGB-oligonucleotide conjugates

Further increases in discriminatory ability of a MGB-oligonucleotide conjugate are obtained when the conjugate also contains a pyrazolo[3,4-d]pyrimidine nucleotide analogue. In this system, the target sequence is located in the *E. coli supF* gene contained in the plasmid pSP189 (Figure 4, SEQ ID No.: 40). See Parris *et al.* (1992) Gene **117**:1-5. Binding sites for the primers used for amplification are indicated as Primer 1 and Primer 2, with Primer 1 having a sequence and polarity that is identical to that shown in Figure 4, and Primer 2 having a sequence and polarity that is the reverse complement to that shown in Figure 4. A 15-mer probe, labeled with fluorescein at the 5'-end, and with TAMRA and CDPI3 at the 3'-end, was designed to be complementary to a region within the approximately 375 nucleotides between the primers, as indicated in Figure 4. This probe was tested using a series of templates, each containing a different single-nucleotide mismatch with the probe sequence, as shown in Figure 4 and described *infra*.

Primer sequences

The forward amplification primer has the sequence:

5' -CTGGGTGAGCAAAAAACAGGAAGGC-3'  
SEQ ID No.: 14

The reverse primer has the sequence:

5' -TGTGATGCTCGTCAGGGG-3'  
SEQ ID No.: 15

Sequence of probe:

The 15-mer probe has the following sequence:

5' -GGGTTCGGACGGGC  
SEQ ID NO.: 16
Template sequences:

The 15-nucleotide region of the template that is complementary to the probe used in this study was modified to generate a series of point mutations, as shown in Figure 4. Each of the mutant templates was used in a separate assay with the 15-mer probe. The mutant sequences within this region of the template were as follows, with the mismatched nucleotide indicated by bold underlining:

\[
\begin{align*}
5' - &GGTTCCCGAGCGGC \quad \text{(perfect match)} \\
5' - &GAGTTCCCGAGCGGC \quad \text{(32 G-A mismatch)} \\
5' - &GGTTTCCCGAGCGGC \quad \text{(36 C-T mismatch)} \\
5' - &GGTTGCCCGAGCGGC \quad \text{(36 C-G mismatch)} \\
5' - &GGTTACCGAGCGGC \quad \text{(36 C-A mismatch)} \\
5' - &GGTTCTCGAGCGGC \quad \text{(37 C-T mismatch)} \\
5' - &GGTTACCGAGCGGC \quad \text{(37 C-A mismatch)} \\
5' - &GGTTCCCGAGCGGC \quad \text{(39 G-C mismatch)} \\
5' - &GGTTCCCGTGCAGGC \quad \text{(40 A-T mismatch)} \\
5' - &GGTTCCCGAAGCGGC \quad \text{(41 G-A mismatch)} \\
5' - &GGTTCCCGACCGGC \quad \text{(41 G-C mismatch)} \\
5' - &GGTTCCGGACAGGCC \quad \text{(43 G-A mismatch)} \\
5' - &GGTTCCGGACGTGC \quad \text{(43 G-T mismatch)} \\
& \\
& \text{SEQ ID NO.: 17} \\
& \text{SEQ ID NO.: 18} \\
& \text{SEQ ID NO.: 19} \\
& \text{SEQ ID NO.: 20} \\
& \text{SEQ ID NO.: 21} \\
& \text{SEQ ID NO.: 22} \\
& \text{SEQ ID NO.: 23} \\
& \text{SEQ ID NO.: 24} \\
& \text{SEQ ID NO.: 25} \\
& \text{SEQ ID NO.: 26} \\
& \text{SEQ ID NO.: 27} \\
& \text{SEQ ID NO.: 28} \\
& \text{SEQ ID NO.: 29} \\
& \text{SEQ ID NO.: 30}
\end{align*}
\]

The assay was conducted according to Wittwer (1997a,b, supra). Figure 5 shows that, when a MGB-conjugated 15-mer, additionally having all guanine residues replaced by the guanine analogue 6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one (ppG), is used in an assay in which annealing/elongation temperature is conducted at 75°C, generation of signal by probes with a single-base mismatch is completely
suppressed, with no effect on the level of signal generated by the perfectly-matched probe.

Thus, the combination of MGB conjugation, substitution with modified nucleotides and appropriate reaction conditions enable facile discrimination between a perfect-matched hybrid and a hybrid containing a single-nucleotide mismatch, at high stringency, allowing a heretofore unparalleled degree of specificity to be obtained in hybridization reactions with short oligonucleotides.

Example 4: Effects of base composition and oligonucleotide backbone on hybrid stability of MGB-oligonucleotide conjugates

MATERIALS AND METHODS

Synthesis of oligonucleotides (ODNs)

All ODNs were prepared from 1 μmol appropriate CPG support on an ABI 394 synthesizer using the protocol supplied by the manufacturer. Protected β-cyanoethyl phosphoramidites of 2'-deoxyribo and 2'-O-methyl ribonucleotides, CPG supports, deblocking solutions, cap regents, oxidizing solutions and tetrazole solutions were purchased from Glen Research. 5'-Aminohexyl modifications were introduced using an N-(4-monomethoxytrityl)-6-amino-1-hexanol phosphoramidite linker (Glen Research). 3'-Aminohexyl and 3'-hexanol modifications were introduced using the CPG prepared as previously described. Petrie et al. (1992) Bioconjugate Chem. 3:85-87. All other general methods employed for preparative HPLC purification, detritylation and butanol precipitation were carried out as described. Reed et al. (1991) Bioconjugate Chem. 2:217-225. All purified octanucleotides were analyzed by C-18 HPLC (column 250 x 4.6 mm) in a gradient of 0-30% acetonitrile in 0.1 M triethylamine acetate buffer, pH 7.0, over 20 min at a flow rate of 2 ml/min. Pump control and data processing were performed using a Rainin Dynamax chromatographic software package on a Macintosh computer. ODN purity was further confirmed by capillary gel electrophoresis (CGE) with a P/ACE™
2000 Series equipped with an eCAP™ cartridge (Beckman, Fullerton, CA). The octanucleotides were >95 % pure by C-18 HPLC and showed one major peak on CGE. Thermal denaturation studies were performed as described. Lukhtanov et al. (1995) *Bioconjugate Chem.* 6:418-426; Lukhtanov et al. (1996) *Bioconjugate Chem.* 7:564-567. The melting temperatures (T_{max} values) of the hybrids were determined from the first derivative maxima (change in A_{260} with respect to time) and are shown in Tables 7-10.

**Synthesis of CDPI₃-tailed ODN conjugates**

Methods for conjugation of the 1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate trimer (CDPI₃) to ODNs have been published. Lukhtanov et al. (1995), *supra*. All (CDPI₃)-tailed octanucleotides were isolated from reaction mixtures and, if necessary, repurified on an analytical (4.6 x 250 mm) PLRP-S column (Polymer Labs) using a gradient of acetonitrile (0-60% for mono- and 0-80% for bis-CDPI₃-tailed ODNs) in 0.1 M triethylammonium acetate, pH 7.5. The column was incubated at 70-75°C. All ODN-CDPI₃ conjugates prepared were analyzed and characterized as described. Lukhtanov et al. (1995), *supra*.

**Molar extinction coefficients of octanucleotides and their derivatives**

The concentrations of the octanucleotides and their derivatives were measured spectrophotometrically. The molar extinction coefficients (ε_{260}) of unmodified octadeoxyribonucleotides were determined by measuring the absorption of the ODNs before and after complete hydrolysis by snake venom nuclease. Shabarova et al. (1981) *Nucleic Acids Res.* 9:5747-5761. With this value, ³²P-labeled CDPI₃-tailed conjugates with known specific activities were prepared and their ε_{260} values determined as described. Lokhov et al. (1992) *Bioconjugate Chem.* 3:414-419. Molar extinction coefficients of all octadeoxyribonucleotides and their CDPI₃ derivatives used in this study are, in order of unmodified ODN, mono-CDPI₃ and di-CDPI₃ derivative: d(pT)₂p, 65.8, 110.1, 178.1/mM/cm; d(pA)₂p, 81.9, 150.0,
218.0/mM/cm; d(pApGpCpGpGpApTpGp), 74.0, 162.9, 230.9/mM/cm; d(pCpApTpCpGpGpCpTp), 65.0, 136.9, 204.9/mM/cm. These extinction coefficients were used to determine the concentration of all other backbone-modified octanucleotides and their CDPI3 conjugates. To calculate $\varepsilon_{260}$ values for deoxyinosine-containing ODNs the value of 4.6/mM/cm multiplied by the number of hypoxanthine bases was subtracted from the extinction coefficients of the corresponding dG-containing ODNs.

RESULTS

CDPI3 residue conjugated to AT-rich duplexes

The antibiotic CC-1065 (Reynolds et al. (1985) Biochemistry 24:6228-6237) and its numerous synthetic derivatives (Boger et al. (1995) Proc. Natl. Acad. Sci. USA 92:3642-3649), including CDPI3, have a strong affinity for AT-rich sites of double-stranded DNA, as do most of the MGBs. CC-1065 also binds to and alkylates AT-rich sequences in RNA-DNA hybrids. Kim et al. (1995) Antisense Res. Dev. 5:149-154. We expected the binding properties of ODN-conjugated CDPI3 to be similar to that observed for free CDPI3. We reported earlier on binding of oligothymidylates carrying a CDPI6 moiety to polyadenylic acids. Lukhanetov et al. (1995, 1996) supra. Here we use the short d(pT)$_8$$\cdot$$d$(pA)$_8$ duplex as a model system for a more comprehensive investigation. NMR analysis of a CC-1065-DNA complex (Seahill et al. (1990) Biochemistry 29:2852-2860) and noncompetitive binding of CDPI3 and ethidium bromide to AT-rich DNA duplexes (Boger et al. (1992) J. Org. Chem. 57:1277-1284) have shown that these compounds span 5-6 base pairs in the DNA minor groove. An octamer duplex, therefore, is a length of double-stranded DNA sufficient to accommodate the conjugated CDPI3 residue and the linkers used in the present study.

A variety of octa-adenylate and octathymidylate derivatives with DNA, 2'-O-methyl RNA or DNA phosphorothioate backbones and carrying CDPI3 residues at
either or both termini were prepared. The structures of CDPI$_3$ and linkers for 5'- and 3'-tailed ODN conjugates are shown in Figure 1. Complementary duplexes constructed from these sequences were melted and the $T_{\text{max}}$ data were shown in Table 2. Although the complex strands were taken in equimolar ratio and only single melting transitions were observed in all cases, the possibility of higher order structure formation, other than duplex, cannot be completely excluded. However, a previous study conducted on d(pT)$_8$$\cdot$poly(dA)/poly(rA) did not reveal a tendency of CDPI$_3$-tailed ODNs to form triplex structures. Lukhtanov et al. (1995) supra.

As expected, the most dramatic stabilization was achieved for AT duplexes with a regular DNA backbone in both strands. The $T_{\text{max}}$ of the weak d(pT)$_8$$\cdot$d(pA)$_8$ complex, 12-14°C under these conditions, was increased to 53-61°C after one of the strands was conjugated to a MGB. Positioning of the MGB on the duplex gave some strand-specific effects. Location of the CDPI$_3$ moiety on either the 3'- or 5'-end of octathymidylate did not significantly affect duplex stability ($T_{\text{max}}$ = 56 or 58°C), but did so when conjugated to oligoadenylate sequences. Octa-adenylate carrying the 3'-CDPI$_3$ residue formed the most stable complementary complex ($T_{\text{max}}$ = 61°C) and the 5'-tailed conjugate the least in this series ($T_{\text{max}}$ = 53°C).

Table 3 shows two examples of longer DNA duplexes with terminal AT-rich sequences, which were also stabilized by tethered CDPI$_3$ residues. These tetradecanucleotides were designed to test the effect of CDPI$_3$ binding in a region of mixed or alternating AT sequences, as opposed to the A$_8$T$_8$ homopolymeric sequence above. Conjugation of CDPI$_3$ to the 3'-end of these ODNs increased $T_{\text{max}}$ values of the complementary duplexes by 21-22°C. Although this value is half that observed for d(pT)$_8$$\cdot$d(pA)$_8$ duplexes (40-49°C), the overall free energy contribution of the CDPI$_3$ residue was estimated and found to be comparable in both cases. The decrease in $\Delta T_{\text{max}}$ was expected, since unmodified hexadecanucleotide duplexes (Table 3) were significantly more stable than d(pT)$_8$$\cdot$d(pA)$_8$ ($T_{\text{max}}$ = 48-49 versus 12-14°C).
Enhancement of nuclease resistance of ODNs by replacement of the phosphodiester group with a phosphorothioate is well established. Eckstein et al. (1970) *Eur. J. Biochem.* 13:558-564; Agrawal et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:7595-7599. This modification generally reduces, however, the affinity of the ODN for a complementary single-stranded target. See, for example, Suggs et al. (1985) *Nucleic Acids Res.* 13:5707-5716; Cosstick et al. (1985) *Biochemistry* 24:3630-3638; LaPlanche et al. (1986) *Nucleic Acids Res.* 14:9081-9093; and Stein et al. (1988) *Nucleic Acids Res.* 16:3209-3221. As we found here, d(pT)₈ • d(pA)₈ duplexes having a phosphorothioate backbone in either the d(pT)₈ strand or both strands were significantly destabilized and showed no melting transition over 0°C (Table 2). The phosphorothioate analog of d(pA)₈ formed a weak hybrid with unmodified d(pT)₈ (Tₘ₉ = 8-9°C). Conjugation of a single CDPI₃ residue increased the Tₘ₉ of all these duplexes into a melting range of 35-56°C, a stabilization effect in some cases of >45°C over the Tₘ₉ of the analogous unmodified complexes. An implication of this finding is that replacement of a phosphate oxygen atom with sulfur does not seem to change the geometry of the minor groove of AT-rich regions, which is still optimal for binding the CDPI₃ moiety.

The tethered CDPI₃ has almost no effect on RNA • RNA duplexes, which are known to adopt the A-form in aqueous solutions and have a very broad minor groove. For example, addition of a CDPI₃ residue to the 3'-end of either strand of a 2'-O-Me-r(pT)₈ • 2'-O-Me-r(pA)₈ duplex showed a modest positive effect on Tₘ₉ of 4-9°C. The geometry of RNA • DNA hybrids is somewhere between the A- and B-duplex configurations and both of the 2'-O-Me-RNA • DNA duplexes studied here showed a substantial level of MGB-assisted stabilization, although with some backbone preference. Tethering the MGB residue to the 2'-O-Me-RNA strand was more beneficial, providing an increase in Tₘ₉ of 18°C for 2'-O-Me-r(pT)₈ • d(pA)₈ and >21-22°C for the d(pT)₈ • 2'-O-Me-r(pA)₈ duplex. In contrast, a lower effect on stabilization (ΔTₘ₉ = 7°C) was found when the CDPI₃ residue was bound to the
d(pA)$_8$ strand. CDPI$_3$-tailed d(pT)$_8$ was unusual in that conjugation of the MGB to the 5'-end of octadeoxythymidylate provided >19°C stabilization for its duplex with 2'-O-Me-r(pA)$_8$, whereas 3'-CDPI$_3$ had almost no effect on stability of this complex. Good agreement of these data with our previously reported results obtained on poly(rA) • d(pT)$_8$ (Lukhtanov et al. (1995, 1996, supra) indicates that addition of a methyl group on a 2'-OH in the minor groove of an RNA • DNA duplex does not substantially alter binding properties of the conjugated CDPI$_3$ residue. Similar results have been obtained for unconjugated CC-1065 bound to AT-rich sites in duplexes with varying backbone structures. Kim et al. (1995) Antisense Res. Dev. 5:49-57.
Table 2. Melting temperatures (±1°C) of duplexes formed by octathymidylate and octa-adenylate with different backbone modifications and CDPI₃ residues attached to different ends.

<table>
<thead>
<tr>
<th>Octa-adenylate derivatives: 3'- and 5'-tails*</th>
<th>Octathymidylate derivatives DNA 3'-HEX</th>
<th>5'-Hex-NH₂</th>
<th>3'-CDPI₃</th>
<th>5'-CDPI₂</th>
<th>5',3'-di-CDPI₃</th>
<th>2'-O-Methyl RNA 3'-HEX-OH</th>
<th>3'-CDPI₃</th>
<th>Phosphorothioate DNA 3'-Hex-NH₂</th>
<th>3'-CDPI₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>3'-Hex-NH₂</td>
<td>14</td>
<td>12</td>
<td>58</td>
<td>58</td>
<td>55</td>
<td>12</td>
<td>30</td>
<td>&lt;0</td>
</tr>
<tr>
<td>DNA</td>
<td>5'-Hex-NH₂</td>
<td>13</td>
<td>13</td>
<td>58</td>
<td>56</td>
<td>44</td>
<td>10</td>
<td>28</td>
<td>&lt;0</td>
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<tr>
<td>DNA</td>
<td>3'-CDPI₃</td>
<td>61</td>
<td>61</td>
<td>71</td>
<td>67</td>
<td>64</td>
<td>19</td>
<td>50</td>
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<tr>
<td>DNA</td>
<td>5'-CDPI₃</td>
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<td>53</td>
<td>63</td>
<td>74</td>
<td>61</td>
<td>17</td>
<td>49</td>
<td>41</td>
</tr>
<tr>
<td>2'-O-Methyl RNA 3'-HEX-OH</td>
<td>60</td>
<td>57</td>
<td>69</td>
<td>68</td>
<td>71</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2'-O-Methyl RNA 3'-CDPI₃</td>
<td>&lt;0</td>
<td>&lt;0</td>
<td>~0</td>
<td>19</td>
<td>-</td>
<td>20</td>
<td>29</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>Phosphorothioate DNA 3'-Hex-NH₂</td>
<td>22</td>
<td>21</td>
<td>54</td>
<td>58</td>
<td>-</td>
<td>24</td>
<td>41</td>
<td>&lt;0</td>
<td>ND*</td>
</tr>
<tr>
<td>Phosphorothioate DNA 3'-CDPI₃</td>
<td>8</td>
<td>9</td>
<td>55</td>
<td>55</td>
<td>-</td>
<td>34</td>
<td>48</td>
<td>&lt;0</td>
<td>&lt;0</td>
</tr>
<tr>
<td>3'-CDPI₃</td>
<td>55</td>
<td>56</td>
<td>70</td>
<td>73</td>
<td>-</td>
<td>43</td>
<td>65</td>
<td>40</td>
<td>57</td>
</tr>
</tbody>
</table>

5* The oligonucleotides with this modification have a terminal phosphate linked to the hydroxy group of 1,6-hexanediol (Hex-OH) or 6-amino-1-hexanol (Hex-NH₂) residues. The structure of the CDPI₃ residue and linkers for 3'- and 5'-oligonucleotide conjugates are shown in Figure 1.

b No melting transition detected.
Table 3. Structure and stability of tetradecanucleotide duplexes modified by a CDPI₃ residue

<table>
<thead>
<tr>
<th>Duplex structure</th>
<th>3'-Tail</th>
<th>$T_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$5' - d \ (GpTpGpTpGpCpApTpApTpApTpAp) - X - 3' \ (\text{SEQ ID No.: 33})$</td>
<td>$X = -O(CH_2)_6NH_2$</td>
<td>49</td>
</tr>
</tbody>
</table>
Table 4. Melting temperatures (±1°C) of GC-rich octanucleotide duplexes with CDPI₃ residues attached to the ends

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3’- and 5’-tails*</td>
<td>3’-Hex-NH₂</td>
<td>5’-CDPI₃</td>
<td>3’-CDPI₃</td>
</tr>
<tr>
<td>DNA</td>
<td>3’-Hex-NH₂</td>
<td>41</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>5’-CDPI₃</td>
<td>58</td>
<td>76</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>3’-CDPI₃</td>
<td>57</td>
<td>78</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>3’,5’-di-CDPI₃</td>
<td>60</td>
<td>BT</td>
<td>72</td>
</tr>
<tr>
<td>2’-O-Methyl RNA</td>
<td>3’-Hex-OH</td>
<td>37</td>
<td>20</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>5’-CDPI₃ and 3’-Hex-OH</td>
<td>44</td>
<td>69</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>5’-CDPI₃</td>
<td>44</td>
<td>71</td>
<td>BT</td>
</tr>
<tr>
<td>Phosphorothioate DNA</td>
<td>Unmodified⁸</td>
<td>32</td>
<td>32</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>5’-CDPI₃</td>
<td>38</td>
<td>67</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>3’-CDPI₃</td>
<td>45</td>
<td>71</td>
<td>74</td>
</tr>
</tbody>
</table>

a Structures of the CDPI₃ residue and linkers for 3’- and 5’-oligonucleotide conjugates are shown in Figure 1.
b These ODNs have no tails.
c No melting transition was detected.
d Melting transition was too broad for Tₘₕ to be accurately determined.
Effect of addition of a MGB residue to a GC-rich octanucleotide duplex

It is well recognized that A/T preference dominates the binding specificity of most MGBs, including CDPI oligomers. This preference is likely due to the hydrophobicity, depth and narrow width of the groove. Together these provide a perfect isothermal and van der Waals fit of the crescent-shaped MGB molecules in the minor groove. Free CDPI₃ was shown to bind not only to poly(dA)·poly(dT) but also to poly(dG)·poly(dC), although with a lower strength. Boger et al. (1992) J. Org. Chem. 57:1277-1284. Therefore, it was interesting to investigate the ability of the CDPI₃ residue to stabilize short GC-rich and mixed duplexes. Table 4 shows the effect of the same MGB modifications discussed above on GC-rich octanucleotide duplexes, in which the nature of the minor groove is altered. The test sequence, with a 3'-hexylamino tail, gave a duplex $T_{\text{max}}$ of 41°C. Addition of a single MGB to either end of strand A increased the $T_{\text{max}}$ by 16-17°C, while addition to strand B gave a smaller increase in $T_{\text{max}}$ ($\Delta T_{\text{max}} = 4-11°C$). Strand B showed a preference for the position of CDPI₃ conjugation, with its 3'-CDPI₃-tailed conjugate forming a more stable duplex ($T_{\text{max}} = 52°C$) than its corresponding 5'-CDPI₃ derivative ($T_{\text{max}} = 45°C$). This effect was seen for all of the other duplexes presented in Table 4 (except when strand B has a 2'-O-methyl backbone) and could be due to the presence of the two AT pairs in the test sequence proximal to the site of conjugation of the MGB.

Addition of the 2'-O-methyl modification (Table 4) to both strands gave a 25°C increase in $T_{\text{max}}$ over the 2'-deoxy strands. This has previously been shown to be a stabilizing modification. Inoue et al. (1987) Nucleic Acids Res. 15:6131-6148. A single MGB tethered to strand B did not change the $T_{\text{max}}$ and addition to strand A gave a modest increase ($\Delta T_{\text{max}} = 2-6°C$). Hybrids between one strand bearing the 2'-O-methyl modification and one with a DNA backbone were similar to those of an unmodified DNA duplex ($T_{\text{max}} = 41°C$), with duplex stability lower when strand A was 2'-O-methyl ($T_{\text{max}} = 37°C$) and higher when strand B was 2'-O-methyl ($T_{\text{max}} = 46°C$). Interestingly, CDPI₃ conjugation destabilized the former of these duplexes.
This effect was observed for both 3’- \( T_{\text{max}} = 29^\circ\text{C} \) and 5’-CDPI\(_3\)-tailed \( T_{\text{max}} = 20^\circ\text{C} \) derivatives of strand B (the DNA strand). This destabilizing effect was exacerbated by phosphorothioate modification of strand B. In both cases, no detectable melting transition over 0°C was detected, compared with a \( T_{\text{max}} \) of 28°C for the non-conjugated counterpart.

Conversion of both backbones of the DNA octameric duplex to all phosphorothioate linkages reduced the \( T_{\text{max}} \) by 17°C (from 41°C to 24°C). Addition of a single MGB to strand B gave little change in \( T_{\text{max}} \) (even a decrease of 8°C in the 5’-CDPI\(_3\) case) and addition to strand A gave a modest increase of 4-12°C. In general, phosphorothioate analogs of strands A and B demonstrated hybridization properties similar to those observed for phosphodiester ODNs except that all of their complementary complexes have lower \( T_{\text{max}} \).

The conjugated CDPI\(_3\) residue stabilized GC-rich DNA duplexes, with the extent of stabilization being about half, in terms of enhancement of \( T_{\text{max}} \), of that observed for the d(pT)\(_8\)·d(pA)\(_8\) complex. In contrast, another type of conjugated MGB, N-methylpyrrole carboxamide (MPC) oligomers, failed to stabilize the same GC-rich octadeoxyribonucleotide duplex used in this study. Sinyakov et al. (1995) J. Am. Chem. Soc. 117:4995-4996. Without wishing to be bound by theory, CDPI\(_3\) may be less sensitive to the structure of the minor groove of a duplex than the netropsin-type MPC peptides, because it does not form any hydrogen bonds with the bases and therefore the interaction is driven by van der Waals contacts or hydrophobic forces. A narrower minor groove may promote better CDPI\(_3\) binding and hence greater duplex stabilization.

**CDPI\(_3\)-conjugated duplexes containing deoxyinosine in place of deoxyguanosine**

Substitution of deoxyguanosine (dG) by deoxyinosine (dI) in the modified ODN could create a minor groove environment more suitable for CDPI\(_3\) binding, as was observed for netropsin and Hoechst 33258 (44). Nielsen (1991) Bioconjugate Chem. 2:1-12; Wartell et al. (1974) J. Biol. Chem. 249:6719-6731; Marck et al.
(1982) *Nucleic Acids Res.* **10**:6147-6161; and Moon *et al.* (1996) *Biopolymers* **38**:593-606. dl-containing analogues of the GC-rich duplex were prepared and studied with respect to MGB-assisted stabilization (Table 5). Replacement of the single dG of strand A with a dl residue gave a 10°C decrease in $T_{\text{max}}$. Addition of a single MGB to the 3'-end of either strand of this complex raised the $T_{\text{max}}$ to a value 7-13°C higher than the parent dG-containing duplex.

The effect of the tethered MGB on the duplex containing four dl residues in strand B was dramatic. The duplex formed between this modified strand B and either the native or dl-substituted analog of strand A was weak ($T_{\text{max}} = 11°C$ for native strand A) or nonexistent (for dl-substituted strand A). Addition of the MGB to the 3'-end of strand A raised the $T_{\text{max}}$ from 11°C to 48°C; while addition of a MGB to the 3'-end of strand B raised the $T_{\text{max}}$ from essentially 0°C to 41°C. These values represent a 37-48°C increase in $T_{\text{max}}$ due to conjugation of a MGB to oligonucleotides forming duplexes containing dl•dC base pairs, which is close to the stability of the analogous dG-containing native strands. This shows that the conjugated CDPI$_3$ residue stabilizes dIdC-rich sequences as well as those rich in dAdT.

**Duplexes with two or more conjugated CDPI$_3$ residues**

d(pT)$_8$·d(pA)$_8$ (Table 2). This is unprecedented for short duplexes with a phosphodiester backbone. The greatest stabilization occurred when either the 5'· or 3'-end of both strands was modified with an MGB; these could bind in the minor groove in an antiparallel mode ($T_{\text{max}} = 71$ and 74°C). The parallel orientation was less beneficial ($T_{\text{max}} = 63$ and 67°C). This is consistent with literature data for ‘free’ MGBs in which only the antiparallel orientation was experimentally observed. Mohan, supra; Fagan, supra; Mrksich, supra; and Animati et al. (1995) J. Med. Chem. 38:1140-1149.

This hyperstabilization did not seem to depend on either sequence or backbone modification. All AT- and GC-rich duplexes that contained two MGB residues, with one conjugated to each of the opposite strands, were substantially stabilized compared with analogous duplexes bearing a single CDPI$_3$ tail (Tables 7 and 9). For example, Table 2 shows that complexes formed by phosphorothioate analogs of d(pT)$_8$ and/or d(pA)$_8$ possessing two CDPI$_3$ residues showed a $T_{\text{max}}$ in the same range ($T_{\text{max}} = 56$-73°C) as their phosphodiester counterparts ($T_{\text{max}} = 63$-74°C). Addition of a MGB to both strands of a duplex with 2’-O-methyl modifications increased the stability by 21°C (from 20°C to 41°C, Table 2). In the case of GC-rich duplexes, the stabilization resulting from conjugation of a second CDPI$_3$ residue to an opposite duplex strand was even greater than that observed for the first CDPI$_3$ incorporation. For instance, attachment of one CDPI$_3$ residue increased stability of the GC DNA duplex by 4-17°C and addition of the second CDPI$_3$ moiety contributed 18-33°C to $T_{\text{max}}$.

The data on duplexes with multiple conjugated MGBs in Tables 7 and 9 show the following trends. If the duplex bore two CDPI$_3$ residues tethered to the same strand at the 3'- and 5'-ends almost no advantage in stability versus the corresponding mono-CDPI$_3$-tailed duplex was seen. This implies a strong hydrophobic interaction between two CDPI$_3$ residues occupying the same site in the minor groove of a short duplex. Furthermore, additional binding between the two MGB moieties attached to
the opposite duplex strands appears to add significantly to hybrid stability. Similar effects of interaction of pendant hydrophobic groups on duplex and triplex stabilization were seen with ODNs conjugated to cholesterol residues. Gryaznov et al. (1993) *Nucleic Acids Res.* **21**:5909-5915. Addition of third and fourth CDPI₃ conjugated residues normally had no or a slightly negative effect on stability of the GC-rich duplexes studied (Table 4).
Table 5. Melting temperatures (±1°C) of dG- and dI-containing octanucleotide duplexes carrying a 3'-CDPI₃ residue

<table>
<thead>
<tr>
<th>Strand A:</th>
<th>Strand B —&gt;</th>
<th>d(AGCGGATG)p</th>
<th>d(AICIIATI)p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3'-Hex-NH₂</td>
<td>3'-CDPI₃</td>
</tr>
<tr>
<td>d(CATCCGCT)p</td>
<td>3'-Hex-NH₂</td>
<td>41</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>3'-CDPI₃</td>
<td>57</td>
<td>81</td>
</tr>
<tr>
<td>d(CATCCICT)p</td>
<td>3'-Hex-NH₂</td>
<td>31</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>3'-CDPI₃</td>
<td>54</td>
<td>79</td>
</tr>
</tbody>
</table>

a Structures of the CDPI₃ residue and linker for the conjugates are shown in Figure 1.
Example 5: Reduced dependence of $T_m$ on base composition of MGB-oligonucleotide conjugates

Short oligonucleotides of differing A+T content, conjugated to a MGB, were used to investigate the effect of base composition on $T_m$ for MGB-short oligonucleotide conjugates (Table 6). $T_m$s were determined on a Perkin Elmer λ2S UV/VIS spectrophotometer, equipped with a PTP-6 temperature controller, using the PECSS software package. The A+T content of the oligonucleotides tested ranged from 12.5% to 100% and $T_m$ was determined for the hybrid of each of the oligonucleotides with its exact complement. As shown in Table 6, 8-mer MGB-oligonucleotide conjugates with A+T contents between 37.5% and 100% had $T_m$s that ranged between 45-54°C, a 9-degree span; while the $T_m$s for unconjugated 8-mers having similar A+T contents ranged over 38°C.

Other experiments have shown that, for 7-mers, $T_m$s of oligonucleotides with A+T contents between 28% and 100% varied over a range of only 4.4°C. These results and those shown in Table 6 suggest that, for short oligonucleotides conjugated to a MGB, $T_m$ is more closely related to length than to base composition.

In addition, the range of $T_m$ values for MGB-conjugated 8-mers extends from 45°C, for oligonucleotides having a base composition of 100% A+T, to 63°C, for 0% A+T. Thus, the $T_m$ values for MGB-conjugated 8-mers range over approximately 18°C. By comparison, the $T_m$ range for unconjugated 8-mers (between 0 and 100% A+T) encompasses at least 52°C (Table 6). There is therefore a clear trend toward lessening of the differences in $T_m$ between short oligonucleotides of different base compositions, when such short oligonucleotides are conjugated to a MGB.

Example 6: Retention of mismatch discriminatory capability of a short oligonucleotide conjugated to a MGB

Although the dependence of $T_m$ on base composition is suppressed for short, 8-mer oligonucleotides that are conjugated to a MGB; their heightened discriminatory
ability, compared to unconjugated oligonucleotides, is retained. Table 7 shows examples of \( T_m \) determinations for an 8-mer MGB-conjugated oligonucleotide hybridized to a perfectly-matched sequence and to four other sequences, each containing a different single-nucleotide mismatch. The minimum difference in \( T_m \) between a perfect match and a single-nucleotide mismatch is 19°C, while the maximum difference is 41°C.
Table 6: Effect of MGB conjugation on T<sub>m</sub> values of 8-mer MGB-oligonucleotide conjugates with varying A+T content

<table>
<thead>
<tr>
<th>Sequence</th>
<th>+ MGB</th>
<th>- MGB</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-MGB-CAGCGGCG</td>
<td>63</td>
<td>52</td>
</tr>
<tr>
<td>5'-MGB-CAGCGACG</td>
<td>53</td>
<td>45</td>
</tr>
<tr>
<td>5'-MGB-CAGTGACG</td>
<td>49</td>
<td>38</td>
</tr>
<tr>
<td>5'-MGB-CAGTGACA</td>
<td>47</td>
<td>33</td>
</tr>
<tr>
<td>5'-MGB-CAITIACA</td>
<td>53</td>
<td>17</td>
</tr>
<tr>
<td>5'-MGB-CAATGACA</td>
<td>54</td>
<td>27</td>
</tr>
<tr>
<td>5'-MGB-CAATGATA</td>
<td>45</td>
<td>20</td>
</tr>
<tr>
<td>5'-MGB-CAATAATA</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>5'-MGB-TAATAATA</td>
<td>45</td>
<td>&lt;0</td>
</tr>
</tbody>
</table>

Table 7: Mismatch discrimination by 8-mer MGB-oligonucleotide conjugates

<table>
<thead>
<tr>
<th>Sequence</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'-TTTTTGTCACTGTTT ACAGTGAC-MGB-5' (SEQ ID NO.:35)</td>
<td>47</td>
</tr>
<tr>
<td>5'-TTTTTGTCATGTTT ACAGTGAC-MGB-5' (SEQ ID NO.:36)</td>
<td>20</td>
</tr>
<tr>
<td>5'-TTTTGTTACTGTTT ACAGTGAC-MGB-5' (SEQ ID NO.:37)</td>
<td>25</td>
</tr>
<tr>
<td>5'-TTTTGACACTGTTT ACAGTGAC-MGB-5' (SEQ ID NO.:38)</td>
<td>28</td>
</tr>
<tr>
<td>5'-TTTTATCACTGTTT ACAGTGAC-MGB-5' (SEQ ID NO.:39)</td>
<td>6</td>
</tr>
</tbody>
</table>
While the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications may be practiced without departing from the spirit of the invention. Therefore the foregoing descriptions and examples should not be construed as limiting the scope of the invention.
CLAIMS

What is claimed is:

1. A method for hybridizing nucleic acids, comprising the steps of:
   (a) providing a first nucleic acid and a second nucleic acid,
   (b) incubating the nucleic acids under hybridization conditions, and
   (c) identifying hybridized nucleic acids;

   wherein at least one of the nucleic acids comprises a minor groove binder (MGB)-oligonucleotide conjugate;

   wherein the minor groove binder is a molecule having a molecular weight of approximately 150 to approximately 2,000 Daltons that binds in a non-intercalating manner into the minor groove of a double-stranded nucleic acid with an association constant of greater than approximately $10^3 \text{M}^{-1}$.

2. The method according to claim 1, wherein the MGB-oligonucleotide conjugate is a probe comprising a detectable label.

3. The method according to claim 2, wherein the detectable label is a fluorescent label.

4. The method according to claim 3, wherein the MGB-oligonucleotide conjugate further comprises an agent that quenches the emission of the fluorescent label.

5. The method according to claim 4, further comprising the step of altering the spatial relationship between the fluorescent label and the agent which quenches the emission of the fluorescent label.

6. The method according to claim 2, wherein the method further comprises the step of releasing label from the probe subsequent to hybridization.

7. The method according to claim 5, wherein the method further comprises the step of releasing label from the probe subsequent to hybridization.

8. The method according to claim 6, wherein release of label occurs as a result of exonuclease hydrolysis.

9. The method according to claim 7, wherein release of label occurs as a result of exonuclease hydrolysis.
10. The method according to claim 3, wherein more than one probe is used.

11. The method according to claim 10, wherein a first and second probe is used.

12. The method according to claim 11, wherein the first probe comprises a fluorescence donor and the second probe comprises a fluorescence acceptor, and further wherein the emission wavelengths of the fluorescence donor overlap the absorption wavelengths of the fluorescence acceptor.

13. The method according to claim 1 wherein the MGB-oligonucleotide conjugate is a primer comprising a free 3'-hydroxyl group.

14. The method according to claim 13, further comprising the step of extending the primer with a polymerizing enzyme.

15. The method according to claim 14, wherein the polymerizing enzyme is a thermostable enzyme.

16. The method according to claim 13, wherein the MGB-oligonucleotide conjugate is a primer in an amplification reaction.

17. The method according to claim 16, wherein the amplification reaction is a polymerase chain reaction.

18. A method for primer extension, comprising the steps of:

(a) providing a sample containing a target sequence,

(b) providing one or more oligonucleotide primers complementary to regions of the target sequence,

(c) providing a polymerizing enzyme and nucleotide substrates, and

(d) incubating the sample, the oligonucleotide primers, the enzyme and the substrates under conditions favorable for polymerization; wherein at least one of the primers comprises a MGB-oligonucleotide conjugate.

19. The method according to claim 18, wherein the method is an amplification reaction.
20. The method according to claim 19, wherein the amplification reaction is a polymerase chain reaction.

21. A method for discriminating between polynucleotides which differ by a single nucleotide, the method comprising the following steps:

(a) providing a polynucleotide comprising a target sequence,
(b) providing at least two MGB-oligonucleotide conjugates, wherein one of the at least two MGB-oligonucleotide conjugates has a sequence that is perfectly complementary to the target sequence and at least one other of the MGB-oligonucleotide conjugates has a single-nucleotide mismatch with the target sequence;
(c) separately incubating each of the MGB-oligonucleotide conjugates with the polynucleotide under hybridization conditions; and
(d) determining the hybridization strength between each of the MGB-oligonucleotide conjugates and the polynucleotide.

22. A method for discriminating between polynucleotides which differ by a single nucleotide, the method comprising the following steps:

(a) providing a MGB-oligonucleotide conjugate of defined sequence,
(b) providing at least two polynucleotides, each of which comprises a target sequence, wherein one of the polynucleotides has a target sequence that is perfectly complementary to the MGB-oligonucleotide conjugate and at least one other of the polynucleotides has a target sequence having a single-nucleotide mismatch with the MGB-oligonucleotide conjugate;
(c) separately incubating each of the polynucleotides with the MGB-oligonucleotide conjugate under hybridization conditions; and
(d) determining the hybridization strength between each of the polynucleotides and the MGB-oligonucleotide conjugate.

23. A method of ligating two or more oligonucleotides, each of which is hybridized to adjacent sites on a target nucleic acid, comprising the steps of:

(a) providing a sample containing a target sequence,
(b) providing at least two oligonucleotides which are
complementary to adjacent sites on the target sequence,

(c) incubating the sample and the oligonucleotides under conditions favorable for ligation, and

(d) identifying ligated nucleic acids;

wherein at least one of the oligonucleotides comprises a MGB-oligonucleotide conjugate.

24. An oligonucleotide probe for use in mismatch discrimination, said probe comprising a 5'-end, a 3'-end and one or more detectable labels, wherein the probe is a MGB-oligonucleotide conjugate.

25. The probe according to claim 24 wherein the detectable label is a fluorescent label.

26. The probe according to claim 25 wherein the label is a fluorescein.

27. The probe according to claim 25 wherein the label is a cyanine.

28. The probe according to claim 25 wherein the label is a rhodamine.

29. The probe according to claim 24 wherein the MGB is located at the oligonucleotide 5' end.

30. The probe according to claim 24 wherein the MGB is located at the oligonucleotide 3' end.

31. The probe according to claim 24 wherein the label is located at the oligonucleotide 5' end.

32. The probe according to claim 24 wherein the label is located at the oligonucleotide 3' end.

33. The probe according to claim 24 wherein the MGB is selected from the group consisting of a trimer of 1,2-dihydro-(3H)-pyrrolo[3,2-e]indole-7-carboxylate (CDPI₃) and a pentamer of N-methylpyrrole-4-carbox-2-amide (MPC₅).

34. The probe according to claim 25 comprising multiple fluorescent labels.

35. The probe according to claim 34 wherein the emission wavelengths of one of the fluorescent labels overlaps the absorption wavelengths of another of the fluorescent labels.
36. The probe according to claim 25 further comprising a quenching agent which quenches the fluorescence emission of the fluorescent label.

37. The probe according to claim 36 wherein the fluorescent label is a fluorescein.

38. The probe according to claim 37 wherein the quenching agent is tetramethylrhodamine.

39. A MGB-oligonucleotide conjugate for use as a primer comprising a 5' end and a 3' end, wherein the 3' end is extendible by a polymerizing enzyme.

40. The primer according to claim 39, wherein the 3' end comprises a free 3' hydroxyl group.

41. The primer according to claim 39, wherein the primer has been extended by a polymerizing enzyme.

42. The primer according to claim 41, wherein the primer has been extended during the synthesis of a cDNA molecule.

43. The primer according to claim 41, wherein the primer has been extended during an amplification reaction.

44. The probe according to claim 24, wherein inosine is substituted for guanosine.

45. The probe according to claim 24, wherein

6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one is substituted for guanine.

46. The probe according to claim 24, wherein

4-amino-1H-pyrazolo[3,4-d]pyrimidine is substituted for adenine.

47. The probe according to claim 24, wherein

1H-pyrazolo[3,4-d]pyrimidin-4(5H)-6(7H)-dione is substituted for adenine.

48. The primer according to claim 39, wherein inosine is substituted for guanosine.

49. The primer according to claim 39, wherein

6-amino-1H-pyrazolo[3,4-d]pyrimidin-4(5H)-one is substituted for guanine.

50. The primer according to claim 39, wherein

4-amino-1H-pyrazolo[3,4-d]pyrimidine is substituted for adenine.
51. The primer according to claim 39, wherein 1H-pyrazolo[3,4-d]pyrimidin-4(5H)-6(7H)-dione is substituted for adenine.
52. A composition comprising the probe according to claim 24.
53. A composition comprising the primer according to claim 39.
54. The probe according to claim 24, wherein the probe is less than 20 nucleotides in length.
55. The primer according to claim 39, wherein the primer is less than 20 nucleotides in length.
56. A kit for amplification comprising one or more primers according to claim 39.
57. A kit for hybridization analysis comprising one or more probes according to claim 24.
58. A kit for use in a hydrolyzable probe assay comprising one or more probes according to claim 24.
59. A kit for use in single nucleotide mismatch detection comprising one or more probes according to claim 24.
60. A kit for use in single nucleotide mismatch detection comprising one or more primers according to claim 39.
61. A kit for use in nucleotide sequence analysis comprising one or more of the probes of claim 24.
62. A kit for use in nucleotide sequence analysis comprising one or more of the primers of claim 39.
63. A method for primer-dependent nucleotide sequence analysis wherein a MGB-oligonucleotide conjugate is used as a primer.
64. A method for determining the sequence of a polynucleotide comprising the steps of:
   (a) providing an array of oligonucleotide probes of different sequences,
   (b) incubating the polynucleotide and the array under hybridization conditions, and
   (c) determining to which of the oligonucleotide probes in
the array the polynucleotide hybridizes;

wherein one or more of the oligonucleotide probes comprises a MGB-oligonucleotide conjugate.

65. A method for examining gene expression comprising the steps of:

(a) providing an array of oligonucleotide probes of different sequences,

(b) incubating a population of polynucleotides with the array under hybridization conditions, and

(c) determining to which of the oligonucleotide probes in the array the population hybridizes;

wherein one or more of the oligonucleotide probes comprises a MGB-oligonucleotide conjugate.

66. A method for identifying one or more mutations in a gene of interest comprising the steps of:

(a) providing an array of oligonucleotide probes of different sequences,

(b) incubating a polynucleotide sample with the array under hybridization conditions, and

(c) determining to which of the oligonucleotide probes in the array the polynucleotide hybridizes;

wherein one or more of the oligonucleotide probes comprises a MGB-oligonucleotide conjugate.

67. The method of hybridization according to claim 1, wherein the melting temperature of the hybridized nucleic acid is independent of base composition.

68. The method of hybridization according to claim 66 wherein the melting temperature of the hybridized nucleic acid is dependent primarily on the length of the hybridized nucleic acid.

69. A MGB-oligonucleotide conjugate that hybridizes to a target nucleic acid to form a hybrid, wherein the melting temperature of the hybrid is independent of base composition.
70. A method for cDNA synthesis, wherein a MGB-oligonucleotide conjugate is used as a primer.

71. A method for detecting a target sequence in a polynucleotide, wherein the polynucleotide is present in a mixture comprising a plurality of polynucleotides that do not contain the target sequence, and wherein the target sequence differs by only a single nucleotide from one or more sequences in the non-target polynucleotides, the method comprising:

(a) providing a Minor Groove Binder (MGB)-oligonucleotide conjugate, wherein the sequence of the oligonucleotide is perfectly complementary to the target sequence;

(b) incubating the MGB-oligonucleotide conjugate and the mixture under hybridization conditions; and

(c) detecting polynucleotides which preferentially hybridize to the MGB-oligonucleotide conjugate, whereby a polynucleotide which preferentially hybridizes to the MGB-oligonucleotide conjugate contains the target sequence.

72. The method according to claim 71 wherein the polynucleotide is DNA.

73. The method according to claim 72 wherein the polynucleotide comprises a coding region.

74. The method according to claim 72 wherein the polynucleotide comprises a non-coding region.

75. The method according to claim 72 wherein the polynucleotide comprises a mutant sequence.

76. The method according to claim 72 wherein the target sequence comprises an allele of a polymorphic sequence.
77. The method according to claim 76 wherein the polynucleotides in the mixture comprise different alleles of the polymorphic sequence.
FIG. 1
4741  AAAACTCTCA AGGATCTTAC GGCTGTTGAG ATCCAGTTCG ATGTAAACCCA CTCGTGCACC

4801  CAACCTGATCT TCAGCATCTT TTACTTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG

4861  GCAAAATGCC GCAAAAAAGG GAATAAGGGG GACACGAAAA TGTTGAATAC TCATACTCTT

SUPF NUCLEOTIDE 1

4921  CTTTTTTCAA TATTTTTGAA GCATTATTCG AAATTCGAGA GCCCTGCTCG

18-MER

21

15-MER

12-MER

AGCTGTGTGGT GGGTCCCAGA GCGGCAAAAG GGAGCAGACT CTAATCTGC CGTCATCGAC

32  A
36  T
36  G
36  A
37  T
37  A
39  C
41  A
41  T
43  A
44  T

81

TTGAGGGTTC AGATCCTTG CCCCCACCC ACGGCGAAA TTTGTTACCC GGATCTCTAG

141

CGAAGCTAA GATTTTTTTT ACGCGTGAGC TCGACTGACT CCNNNNNNNNN GAGCTCAATT

201

CGGTGAGGT CCGGCGCGGT TGCTGGCCTT TTTCATAGTG CTCGCCCCCC GCCGCCGCAGCA

FIG. 4
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Epoch Pharmaceuticals

(ii) TITLE OF INVENTION: HYBRIDIZATION AND MISMATCH DISCRIMINATION USING OLIGONUCLEOTIDES CONJUGATED TO MINOR GROOVE BINDERS

(iii) NUMBER OF SEQUENCES: 40

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: MORRISON & FOERSTER
(B) STREET: 755 PAGE MILL ROAD
(C) CITY: PALO ALTO
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94304-1018

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: Windows
(D) SOFTWARE: FastSEQ for Windows Version 2.0b

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Brennan, Sean M
(B) REGISTRATION NUMBER: 39,917
(C) REFERENCE/DOCKET NUMBER: 34469-20004.40

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 650-813-5600
(B) TELEFAX: 650-494-0792
(C) TELEX: 706141

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

(2) INFORMATION FOR SEQ ID NO:2:

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SUBSTITUTE SHEET (RULE 26)
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

(2) INFORMATION FOR SEQ ID NO:3:

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

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

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TAATAACGTT

(2) INFORMATION FOR SEQ ID NO:7:

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

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

TAATAACGTT

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

CGGGCAAAA

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

CGGGCAAGG ATTTAA

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

GGCAAAA

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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

CGGCTCTA

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGGCTCTAAT CTATTA

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATTTTAGAT AACCTT

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTGGGTGAGC AAAAAACAGGA AGGC

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGTGATGCTC GCAGGGGGG

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(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
GGGTTCGGA GCGGC

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:
GGGTTCGGA GCGGC

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
GAGTTTCGGA GCGGC

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
GGGTTCGGA GCGGC

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGGTGCCGA GCGGC

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGGTACCGA GCGGC

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GGGTTCGGA GCGGC

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGGTTCAGA GCGGC

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGGTCCCCCA GCGGC

(2) INFORMATION FOR SEQ ID NO:25:
(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:25:**

GGGTCCCAGT GCGGC

(2) **INFORMATION FOR SEQ ID NO:26:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:26:**

GGGTCCCAGA ACGGC

(2) **INFORMATION FOR SEQ ID NO:27:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:27:**

GGGTCCCAGA CCGGC

(2) **INFORMATION FOR SEQ ID NO:28:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:28:**

GGGTCCCAGA GCAGGC

(2) **INFORMATION FOR SEQ ID NO:29:**

(i) **SEQUENCE CHARACTERISTICS:**
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) **SEQUENCE DESCRIPTION: SEQ ID NO:29:**
GGGTCCCGA GCTGC

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GGGTCCCGA GCGTC

(2) INFORMATION FOR SEQ ID NO:31:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GTGTGTCATA TATA

(2) INFORMATION FOR SEQ ID NO:32:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TATATATGAC ACAC

(2) INFORMATION FOR SEQ ID NO:33:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GTGTGTCATA AATA

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TATTTATGAC ACAC

(2) INFORMATION FOR SEQ ID NO:35:

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

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

TTTTGTCACT GTTT

(2) INFORMATION FOR SEQ ID NO:36:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

TTTTGTCATT GTTT

(2) INFORMATION FOR SEQ ID NO:37:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

TTTTGTTACT GTTT

(2) INFORMATION FOR SEQ ID NO:38:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TTTTGACACT GTTT

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SUBSTITUTE SHEET (RULE 26)
(2) INFORMATION FOR SEQ ID NO: 39:

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

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:
TTTATCAGT GTTT

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 510 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:
AAAACTCTCA AGGATCTTAC CGCTGTTGAG ATCCAGTTTCG ATGTAACCCA CTGCTGCACC 60
CAACTGATCT TCAGCATCTT TTACTTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG 120
GCAAAAGGCC GCAAAAAAGG GAATAAGGCC GACACGAAA TGTTGAATAC TCTACCTCTT 180
CCTTTTTCGAA TATATCTGAA GCATTTATCA GGGAAATTCA GAGCCCTGCT CGAGCTGTGG 240
TGGGTTTCGCC GAGGGGCCAA AGGGAGCAGA CTCTAAATCT GCGTCACTCG ACTTCAAGGG 300
TTCGAATCTCT TCCCCACCCA CCACGCGCCG AATTCGGTPAC CCGGATCCTT AGCGAAAGCT 360
AAGATTTTTTT TTACCCGGGTA GCTCGACTGTA CTCCNNNNNN NNGAGCTCAA TTCGGTTCGAG 420
GTCCGGGCGGC GTGCTGGGCG CTGGGCTCATA GGCTCAGGCC CCCTGAGCAG CATCACAAA 480
ATCGACGCTC AAGTCAGAGG TGCGCAAAACC 510

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