

Design of LNA probes that improve mismatch discrimination

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ABSTRACT

Locked nucleic acids (LNA) show remarkable affinity and specificity against native DNA targets. Effects of LNA modifications on mismatch discrimination were studied as a function of sequence context and identity of the mismatch using ultraviolet (UV) melting experiments. A triplet of LNA residues centered on the mismatch was generally found to have the largest discriminatory power. An exception was observed for G–T mismatches, where discrimination decreased when the guanine nucleotide at the mismatch site or even the flanking nucleotides were modified. Fluorescence experiments using 2-aminopurine suggest that LNA modifications enhance base stacking of perfectly matched base pairs and decrease stabilizing stacking interactions of mismatched base pairs. LNAs do not change the amount of counterions (Na^+) that are released when duplexes denature. New guidelines are suggested for design of LNA probes, which significantly improve mismatch discrimination in comparison with unmodified DNA probes.

INTRODUCTION

Recognition of mismatches in DNA or RNA duplexes, e.g. single nucleotide polymorphisms (SNPs), is important in a variety of methods employed in molecular biology and clinical diagnostic, including real-time PCR (1–5), microarrays (5,6), RNA interference (7), antisense inhibition (8) or nanosensors (9). Mismatch discrimination often relies upon differential hybridization between perfectly matched and mismatched duplexes. The relative sensitivity of this approach can be estimated from differences in melting temperatures (T_m) between these species. The ΔT_m is usually small (0.5–3°C) for a single mismatch within a ‘typical’ 25 bp long probe (1), which significantly limits the utility of hybridization-based methods. Use of shorter probes can improve the ΔT_m of a

mismatch assay but can be less specific when used in complex nucleic acid samples.

Recently, chimeric probes having select positions modified with locked nucleic acids (LNAs) were reported to enhance both duplex stability and mismatch discrimination (1,2,4–6, 10–14). LNA monomers contain a modified ribose moiety and are grossly similar to 2'-*O*-methyl RNA. However, *O*-methyl group is further constrained in LNA residues and bridges 2' and 4' carbons of the ribose ring (10). This covalent bridge effectively ‘locks’ the ribose in the N-type (3'-endo) conformation that is dominant in A-form DNA and RNA. This conformation enhances base stacking and phosphate backbone pre-organization (15) and results in improved affinity for complementary DNA or RNA sequences (higher T_m).

LNA analogs have other useful properties. Since water solubility of LNAs is similar to solubility of DNAs or RNAs, modified oligomers can substitute native nucleic acids in many biological applications. LNAs are synthesized using traditional phosphoramidite reagents and are commercially available. Since LNA, DNA and RNA bases can be positioned wherever desired within a sequence, the functional properties of a probe can be altered and adjusted as needed. Further, LNA residues confer a relative degree of nuclease resistance, both by exo- and endonucleases (16). The stability of chimeric LNA/DNA oligomers in serum is comparable to that of phosphorothioate-modified DNA oligomers (17). If several LNA residues are placed at the 5' or 3' termini of the oligomer, digestion by exonucleases is reduced. End-modified gapped chimeric LNA/DNA oligomers have shown favorable properties for use as antisense compounds.

In spite of these superior properties, more widespread use of chimeric LNA probes is limited by the absence of any established thermodynamic characterization of their mismatch discrimination behavior. Probe design is not straightforward since LNA effects are sequence specific, vary with the identity of the mismatch, length of the probe, sequence context and modification pattern. The availability of improved design rules would increase utility of LNAs in hybridization assays. We conducted ultraviolet (UV) melting experiments and evaluated thermal stability for 12 sets of LNA–DNA duplexes with and without mismatches. Various positions of LNA bases relative

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to the mismatch site were systematically investigated. Improved design rules for LNA probes are suggested.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

Oligomers were synthesized using LNA and DNA phosphoramidite chemistry at Integrated DNA Technologies (Coralville, IA). LNA phosphoramidites were obtained from Exiqon (Vedbaek, Denmark). Oligonucleotides were purified using either 8 M urea denaturing PAGE or reverse phase high-performance liquid chromatography (HPLC) as described (18). Oligonucleotide quality was assessed by mass spectrometry and capillary electrophoresis. Electrospray-ionization liquid chromatography mass spectroscopy (ESI-LCMS) was carried out for all purified samples using an Oligo HTCS system (Novatia, Princeton, NJ). Experimental molar masses for all single strand oligomers were within 2 g/mol of expected molar masses. These results confirmed identity of the oligonucleotides. Capillary electrophoresis analysis was done using a Beckman PACE 5000 or Beckman MDQ and all oligonucleotides employed were at least 90% pure.

UV melting experiments

Samples were melted in a buffer (19) containing 1 M NaCl, 10 mM sodium phosphate, 1 mM Na₂EDTA, adjusted with NaOH to pH 7.0. Total Na⁺ concentration was 1.02 M. Buffers were prepared from solid compounds. Some duplexes were melted in lower NaCl/sodium phosphate/Na₂EDTA buffers (19) with total Na⁺ concentrations of 69, 119, 220 and 621 mM. Several duplex sets were also melted in a magnesium buffer containing 2 mM MgCl₂, 10 mM potassium cacodylate, adjusted with cacodylic acid to pH 7.0. This buffer mimics a PCR buffer, however, its pH is significantly less dependent on temperature than the Tris-based buffers more commonly employed in PCR. Magnesium concentrations were verified using EDTA titrations (18). Concentrated stocks of single strand oligomers were dialyzed against low salt 69 mM Na⁺ melting buffer and DNA concentrations were determined from UV absorbance (19). Extinction coefficients of single strand oligonucleotides were calculated from the published nearest-neighbor model (20). Extinction coefficients of LNA and DNA bases were assumed to be the same. Single strand oligomers were mixed in 1:1 molar ratio and diluted with 1 M Na⁺ buffer to a final total single strand concentration, C_t, of 2 μM. Duplex samples intended for the magnesium buffer were desalted by dialysis in 28-well Microdialysis System (Invitrogen, Carlsbad, CA), lyophilized in Speed-Vac concentrator and rehydrated with the magnesium buffer.

UV melting experiments were conducted and analyzed as described earlier (19) using a Beckman DU 650 spectrophotometer with Micro Tm analysis accessory. Absorbance at 268 nm was recorded every 0.1°C at a heating rate of 25°C/h and temperatures were measured by an internal probe located inside of the Peltier holder. Samples in 10 mm pathlength cuvettes were overlaid with 10 μl of mineral oil to prevent evaporation and to make baselines more reproducible. Melting profile of a buffer was subtracted from raw melting curves of DNA samples. The fraction of melted

base pairs, θ , was calculated from the standard formula, $\theta = (A - A_L)/(A_U - A_L)$, where A , A_L and A_U are sample absorbance, absorbance of the lower baseline and absorbance of the upper baseline, respectively. Melting curves were smoothed using a digital filter (21). T_m is defined (22) as the temperature where $\theta = 0.5$. Average error of experimental melting temperatures was $\pm 0.4^\circ\text{C}$. From three to eight melting curves were collected for each DNA duplex oligomer in different cuvettes and in different positions of the Peltier holder to minimize systematic errors. Absorbance of the upper baseline extrapolated to 25°C was used to calculate C_t and to verify DNA concentrations. If any C_t values differed more than 10% from the expected 2 μM concentration, the data were discarded and melting experiments were repeated with fresh samples.

Fluorescence experiments

Fluorescence measurements were conducted using PTI Fluorescence System (Photon Technology International, Birmingham, NJ) and analyzed with FeliX™ software (version 1.4) supplied by the manufacturer. Samples were equilibrated at 12°C and excited by 305 nm light. Bandwidths were set to 4 nm. Each single strand had a concentration of 10 μM and was dissolved in 1 M NaCl, 10 mM sodium phosphate, 1 mM Na₂EDTA melting buffer (pH 7.0).

RESULTS AND DISCUSSION

Evaluation of mismatch discrimination

There is no obvious ‘best way’ to measure and report mismatch discrimination. Every duplex with a single base mismatch can be compared to two different perfectly matched duplexes. For example, a probe-target duplex having an A•C mismatch could be compared with perfectly matched duplexes containing either an A•T or a G•C base pair at that position. Within this study, we used the average T_m of both of these perfectly matched duplexes as the reference T_m and defined the mismatch discriminating temperature difference, $\Delta_{\text{md}}T_m$, as the difference between average melting temperature of the two possible ‘parent’ duplexes and the melting temperature of the mismatch duplex. Perfectly matched ‘parent’ duplexes are obtained when one of two bases at the mismatch site is replaced to create Watson–Crick base pair. For example, mismatch discriminating temperature difference for A•C mismatch is,

$$\Delta_{\text{md}}T_m(\text{A}\bullet\text{C}) = \frac{T_m(\text{A}\bullet\text{T}) + T_m(\text{G}\bullet\text{C})}{2} - T_m(\text{A}\bullet\text{C}), \quad 1$$

where $T_m(\text{A}\bullet\text{C})$ is the melting temperature of the duplex with A•C mismatch. $T_m(\text{A}\bullet\text{T})$ and $T_m(\text{G}\bullet\text{C})$ are the melting temperatures of two perfectly matched duplexes when cytosine base of the mismatch base pair is replaced with thymine, and adenine base of the mismatch base pair is replaced with guanine, respectively. Mismatch discriminating free energy, enthalpy and entropy changes accompanying melting transitions can be similarly defined.

Mismatch discrimination of LNA probes

Sets of sequences are presented in Figure 1. ‘Probe’ sequences contained LNA bases at the positions indicated and were

Set	Sequence (5' to 3')	Description
1DNA	g g t c c t t x c t t g g t g	Unmodified DNA probe
1T	g g t c c t t X c t t g g t g	Triplet of LNA bases
1M	g g t c c t t X c t t g g t g	LNA modification at the <u>m</u> ismatch site
1NN	g g t c c t t X c t t g g t g	LNA modifications at <u>n</u> earest- <u>n</u> eighbor bases of the mismatch
1NNN	g g t c c T t X c t t g g t g	LNA modifications at <u>n</u> ext- <u>n</u> earest- <u>n</u> eighbor bases of the mismatch
1MA	g g t c c t t X c t t g g t g	LNA modifications at the <u>m</u> ismatch and the base <u>a</u> fter mismatch
1BM	g g t c c t T X c t t g g t g	LNA modifications at the base <u>b</u> efore mismatch and at the <u>m</u> ismatch
1ES	g G t C c T t X c T t G g T g	LNA modifications at <u>e</u> very <u>s</u> econd base
1ET	g G t c C t t X c t T g g T g	LNA modifications at <u>e</u> very <u>t</u> hird base
2DNA	c g g t c a t c x a c g t a g c x a g g t c a	Unmodified DNA probe
2T	c g g t c a t c X A c g t a g C X A g g t c a	Triplet of LNA bases
2M	c g g t c a t c X a c g t a g c X a g g t c a	LNA modifications at the <u>m</u> ismatch sites
2MA	c g g t c a t c X A c g t a g c X A g g t c a	LNA modifications at the <u>m</u> ismatch and the base <u>a</u> fter mismatch
2NN	c g g t c a t c X a c g t a g c X A g g t c a	LNA modifications at <u>n</u> earest- <u>n</u> eighbor bases of the mismatch
3DNA	g t g a x a t g c	Unmodified DNA probe
3M	g T g a X a T g c	LNA <u>m</u> odified probe

Figure 1. Sets of DNA and LNA sequences. Mismatch sites 'x' are highlighted in red, DNA bases are lowercase, LNA monomers are uppercase and are underscored with a gray box for easy identification. Uppercase letter C denotes 5-methylcytosine LNA residue. Abbreviated names of sets were created based on sequence number and descriptions.

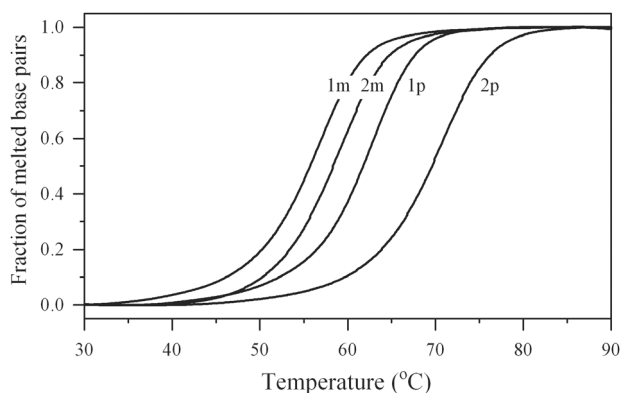


Figure 2. Experimental melting profiles of perfectly matched C•G (1p) and mismatched A•G (1m) duplexes for native 1DNA Set are plotted with melting profiles of isosequential C•G (2p) and A•G (2m) duplexes of the 1T Set, which contains LNA triplets at the mismatch site.

hybridized to unmodified DNA oligomers. Sequence Sets 1, 2 and 3 contained centrally placed mismatches in the context of the trinucleotides -txc-, -cxa- and -axa-, respectively. For designs in Sets 1 and 2, each 'Set' includes 16 distinct duplexes comprising all possible match (4) and mismatch (12) base pairs at the 'x' position. Data for sequence Sets 3 were obtained from Koshkin *et al.* (10). Because the probes have different numbers of base pairs and different sequence context within their mismatch positions, it was possible to discern 'general effects' that are not specific to a given sequence context.

Examples of experimental melting profiles are plotted in Figure 2 for DNA–DNA and LNA–DNA duplexes. Melting profiles of all duplexes displayed single, cooperative

transitions. Heating and cooling melting curves were reversible confirming that transitions were at equilibrium. Figure 2 illustrates that introduction of LNA modifications stabilizes the perfectly matched duplex more than the mismatched duplex (compare differences between 1p, 1m and 2p, 2m curves), which results in improved mismatch discrimination potential. Melting temperatures of more than 300 duplexes were determined and are reported in Tables 1 and 2 and in the Supplementary Data. Mismatch discrimination varies with position of LNA residues and base identity. Significant differences were observed. For example, comparison of Sets 1DNA and 1T in Table 2 shows that LNA modifications increase $\Delta_{md}T_m$ value of an A•A mismatch from 8.4 to 12.3°C, while discrimination of a G•T mismatch decreases from 6.3 to 5.5°C.

Comprehensive analysis of discrimination for 12 kinds of mismatched base pairs is presented in Figure 3 where differences of mismatch discrimination between LNA-modified [$\Delta_{md}T_m(\text{LNA})$] and unmodified DNA [$\Delta_{md}T_m(\text{DNA})$] probes are graphically displayed. Neighboring bases can influence discrimination. The majority of mismatches exhibit positive or insignificant differences of $\Delta_{md}T_m(\text{LNA}) - \Delta_{md}T_m(\text{DNA})$ values, indicating that the addition of LNA bases either improves mismatch discrimination or is neutral. An important exception is the G•T mismatch where LNA modifications of guanine (at the mismatch) or even at neighboring residues are detrimental and decrease mismatch discrimination. This problem can be minimized if G•T mismatch assays are designed to detect the complementary strand, where the LNA probe contains the thymine residue of the mismatch. Alternatively, LNA nucleotides can be introduced two or more bases away from the mismatch guanine residue where they would at least not worsen mismatch discrimination.

Table 1. Melting temperatures, T_m , (°C) and mismatch discriminating temperature differences, $\Delta_{md}T_m$, of native and LNA-modified duplexes based on the sequence Set 1 in 1 M Na⁺ buffer^a

X•Y base pair	Set 1DNA		Set 1T		Set 1M		Set 1MA		Set 1NN		Set 1NNN	
	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$
Perfectly matched duplexes												
A•T	59.4	—	67.5	—	60.9	—	63.6	—	65.7	—	63.1	—
T•A	60.5	—	67.1	—	62.9	—	65.3	—	65.8	—	64.1	—
C•G	61.8	—	69.5	—	65.6	—	67.4	—	68.1	—	65.6	—
G•C	63.9	—	71.4	—	65.1	—	67.8	—	69.5	—	66.9	—
Single base mismatched duplexes												
A•A	51.5	8.4	55.0	12.3	50.6	11.3	52.1	12.3	55.3	10.4	54.0	9.6
A•C	50.3	11.3	55.8	13.7	51.0	12.0	52.4	13.3	54.8	12.8	53.2	11.8
A•G	55.7	4.9	58.3	10.2	52.7	10.5	54.1	11.4	59.6	7.4	57.2	7.1
C•A	49.1	12.0	53.1	15.3	51.3	12.9	51.6	14.8	52.6	14.4	52.2	12.6
C•C	46.8	16.0	49.6	20.9	48.3	17.0	47.6	20.0	49.3	19.5	50.2	16.0
C•T	49.4	11.2	52.8	15.7	51.4	11.8	51.4	14.1	52.6	14.3	53.5	10.8
G•A	53.6	8.6	57.7	11.6	52.0	12.0	53.9	12.7	57.8	9.9	55.4	10.1
G•G	56.1	6.7	60.5	10.0	55.0	10.3	57.1	10.5	62.5	6.3	60.2	6.1
G•T	55.3	6.3	64.0	5.5	58.6	4.4	60.5	5.2	62.5	5.1	58.9	6.0
T•C	49.7	12.5	54.0	15.3	50.9	13.1	52.3	14.2	53.0	14.6	53.8	11.7
T•G	54.3	6.9	59.4	8.9	55.5	8.7	57.2	9.1	57.9	9.1	58.1	6.8
T•T	50.5	9.5	56.1	11.2	52.9	9.0	54.4	10.1	53.9	11.9	54.7	8.9

^aTotal strand concentrations, C_t , were 2 μ M. Values of $\Delta_{md}T_m$ were calculated from nonrounded values of T_m .

Table 2. Melting temperatures, T_m , (°C) and mismatch discriminating temperature differences, $\Delta_{md}T_m$, of native and LNA-modified duplexes based on the sequence Set 2 in 1 M Na⁺ or 2 mM Mg²⁺ buffers^a

X•Y base pair	Set 2DNA in 1 M Na ⁺		2 mM Mg ²⁺		Set 2T in 1 M Na ⁺		2 mM Mg ²⁺		Set 2M in 1 M Na ⁺		Set 2NN in 1 M Na ⁺	
	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$	T_m	$\Delta_{md}T_m$
Perfectly matched duplexes												
A•T	75.8	—	67.3	—	86.4	—	77.6	—	77.3	—	83.8	—
T•A	74.1	—	66.1	—	85.5	—	76.6	—	77.3	—	83.5	—
C•G	77.7	—	69.9	—	90.6	—	82.7	—	81.8	—	87.1	—
G•C	78.8	—	70.5	—	89.0	—	79.4	—	80.7	—	87.3	—
Single base mismatched duplexes												
A•A	64.2	10.7	56.9	9.8	72.5	13.4	64.7	12.4	64.0	13.3	70.7	13.0
A•C	61.8	15.5	55.5	13.5	72.0	15.7	65.0	13.6	63.2	15.8	69.6	15.9
A•G	66.6	10.1	59.7	8.9	73.2	15.3	66.4	13.8	64.5	15.0	72.1	13.3
C•A	61.2	14.7	54.9	13.1	70.7	17.3	63.3	16.4	63.6	15.9	67.4	17.9
C•C	58.6	19.7	53.3	16.9	67.7	22.1	61.1	19.9	61.3	20.0	64.0	23.2
C•T	63.2	13.5	54.7	13.9	71.6	16.9	63.6	16.6	65.3	14.2	68.0	17.4
G•A	66.7	9.7	59.8	8.6	69.2	18.0	61.5	16.5	65.3	13.7	70.8	14.7
G•G	65.4	12.9	57.9	12.3	70.8	19.0	62.2	18.8	65.7	15.5	72.0	15.2
G•T	68.6	8.7	61.2	7.7	79.0	8.7	70.3	8.3	72.8	6.2	78.8	6.7
T•C	63.0	13.4	53.6	14.7	66.5	20.7	58.0	20.0	61.7	17.3	67.4	18.0
T•G	67.5	8.4	60.3	7.7	74.6	13.5	66.8	12.9	69.6	9.9	74.2	11.1
T•T	66.0	9.0	57.9	8.8	71.8	14.2	62.7	14.4	66.6	10.7	72.2	11.5

^a $C_t = 2 \mu$ M. Values of $\Delta_{md}T_m$ were calculated from nonrounded values of T_m .

To find the best modification pattern, Set 1T (triplet of LNA bases), 1M (LNA only at the mismatch site) and 1NN (LNA at nearest-neighbor bases) were compared (Figure 3A and Table 1). The largest $\Delta_{md}T_m(\text{LNA}) - \Delta_{md}T_m(\text{DNA})$ differences are consistently seen in the 1T Set. When the LNA triplet at the mismatch site is reduced to doublets (Sets 1MA and 1BM), the discriminatory power is generally reduced compared to the 1T Set. When only the mismatched nucleotide is LNA-modified (Set 1M), the best discrimination is achieved when the LNA-modified bases are purines. If nearest-neighbor residues are LNAs (Set 1NN), the largest discriminatory power is observed when probes contain a pyrimidine base at the mismatch site. These results agree with the genotyping study of Chou *et al.* (4), which employed LNA probes to detect

a C•A mismatch resulting from 1691G > A mutation in the Factor V Leiden gene. They reported that LNA probes exhibited the highest discriminatory power when a nucleotide flanking cytosine residue of the mismatch site was modified. Examination of Sets 2T, 2M, 2MA and 2NN gives similar results (Figure 3C and Table 2) in spite of the fact that probes for these Sets employ a different sequence. The triplet LNA design (Set 2T) again has the highest discriminatory power. Mismatch discrimination for Sets 2M and 2NN follows similar dependence on purine and pyrimidine bases, although Set 2NN also shows improved mismatch discrimination for some purine mismatches. Therefore, with the exception of a G•T mismatch, the best discrimination is achieved with probes containing a triplet of LNA-modified

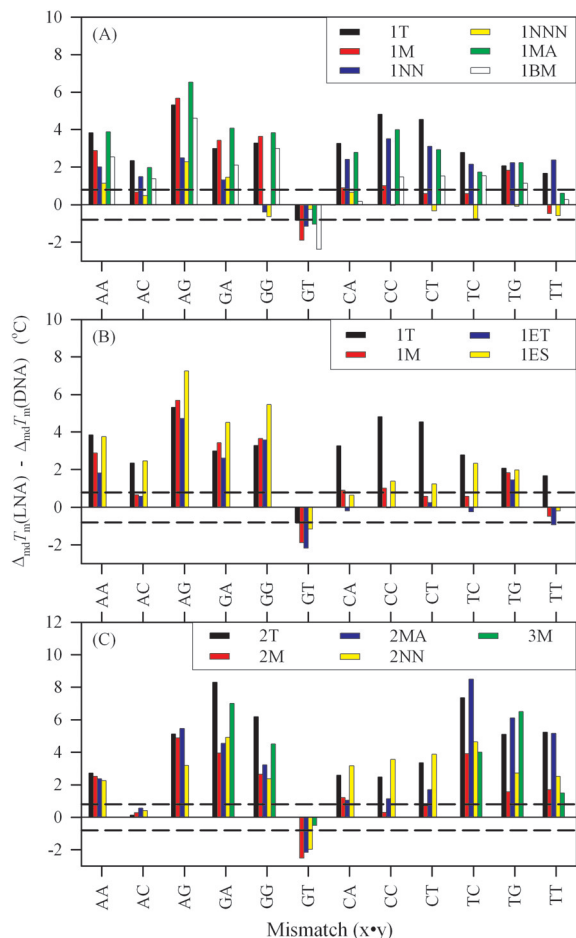


Figure 3. Difference of $\Delta_{\text{md}}T_m$ values between LNA and DNA probes for various mismatches. Sequence Set 1 (panel A and B), and Sets 2 and 3 (panel C) are plotted. Positions of LNA residues and set names are shown in Figure 1. A positive difference indicates improved mismatch discrimination for the LNA probe relative to the DNA probe. Dashed lines denote the range ($\pm 0.8^\circ\text{C}$), which is within the experimental error of the measurements.

nucleotides where the middle base of the triplet is at the mismatch site.

The beneficial effect of LNA modification decreases with distance. Set INNN places LNA residues at the next-nearest-neighbor position (Figure 3A) and differences of $\Delta_{\text{md}}T_m$ values between LNA and DNA probes are mostly within the limits of experimental error. Therefore, the LNA modifications of next-nearest-neighbor nucleotides have little effects on mismatch discrimination. The thermodynamic effects of isolated LNA substitutions are local and do not extend beyond bases immediately flanking the modifications. Because similar $\Delta_{\text{md}}T_m$ values are observed for 1M and 1ET Sets, this is also true for probes where every third base of the probe is modified with LNA (Figure 3B). These results also suggest that the nearest-neighbor model is adequate to predict duplex thermodynamics for LNA-containing sequences. When the fraction of nucleotides modified with LNA is higher, e.g. Set 1ES where every second nucleotide is a LNA, synergistic energetic effects are observed; values of $\Delta_{\text{md}}T_m$ are different in 1M and 1ES Sets. Kierzek *et al.* (12) found LNA effects to be approximately additive when every second base is LNA-modified in

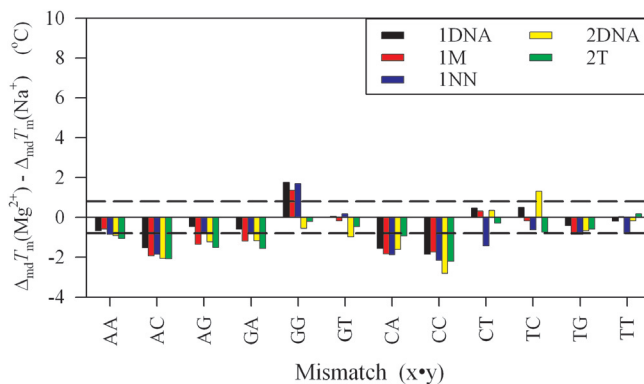


Figure 4. Differences of $\Delta_{\text{md}}T_m$ values between 2 mM Mg^{2+} and 1 M Na^+ buffers for unmodified and LNA-modified probes. Dashed lines denote the range ($\pm 0.8^\circ\text{C}$), which is within the experimental error of the measurements. See Figure 1 for sequences and LNA modification patterns.

2'-*O*-methyl RNA/RNA duplexes. Our results for LNA–DNA duplexes suggest that this additivity may break down when the density of LNA residues is lower. NMR experiments have shown (15,23,24) that the structure of the duplex and sugar puckering are altered surrounding a LNA base and that this can impact thermodynamic parameters for both neighboring DNA and LNA residues. However, these structural effects are local and only the nucleotides immediately flanking a LNA modification are affected (15). The most pronounced structural changes were seen for the nucleotide that follows the LNA residue in 3' direction (15). This is in agreement with our observations that Set 1MA enhances mismatch discrimination more than Set 1BM (Figure 2). Thermodynamic and structural changes are related.

In summary, a triplet of LNA modifications positioned centered at the mismatch site gives the highest difference of melting temperatures between matched and mismatched LNA–DNA duplexes. As an alternative strategy, a LNA modification can be selectively placed at a purine nucleotide in a mismatch site and will also improve mismatch discrimination. If a pyrimidine base is present at the mismatch site, nearest-neighbor nucleotides flanking the pyrimidine base should be modified. An important exception to these rules is the G•T mismatch where any LNA modifications of the guanine nucleotide or either of its nearest-neighbor bases should be avoided.

Effects of Na^+ and Mg^{2+} ions on duplex stability and mismatch discrimination

Because probe hybridizations are done in buffers of various composition, we studied whether different ionic environments could influence these results. Microarray experiments use buffers containing relatively low concentrations of sodium salts in the final wash steps. PCR and other enzymatic experiments commonly employ buffers where magnesium concentrations range from 1 to 5 mM. Therefore, several sequence sets were melted in a 69 mM Na^+ buffer (19) and in a 2 mM Mg^{2+} buffer and mismatch discrimination was compared with the original melting data collected in 1M Na^+ buffer. Figure 4 plots the differences observed in mismatch discrimination between sodium and magnesium buffers. In most cases, the $\Delta_{\text{md}}T_m(\text{Mg}^{2+}) - \Delta_{\text{md}}T_m(\text{Na}^+)$ values are negligible, i.e. the

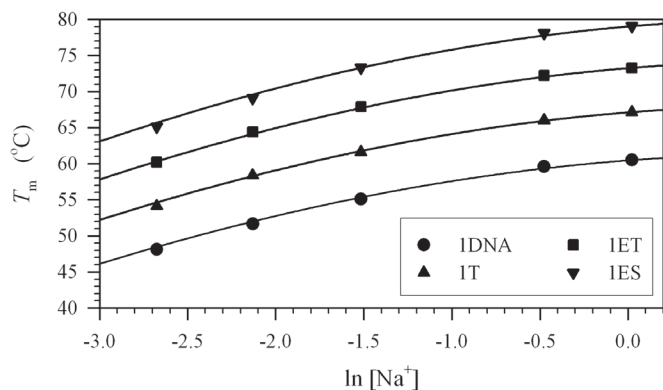


Figure 5. Effects of sodium ions on melting temperatures of DNA–DNA and LNA–DNA duplexes. Duplexes were investigated that had a T•A base pair in the X•Y mismatch site. All duplexes had the same base sequence, 5′-ggctcttctgtg-3′/3′-ccaggaagaaccac-5′, where LNA modifications were introduced at various positions (Figure 1). Solid lines were calculated using a published T_m salt correction (19).

mismatch discrimination in Na^+ and Mg^{2+} buffers are similar for the same probes (see also Table 2 and the Supplementary Data). Probes for the most destabilizing mismatches, A•C and C•C, exhibit slightly smaller discrimination power in the Mg^{2+} buffer than in the 1 M Na^+ buffer. However, similar decreases of mismatch discrimination are observed for both unmodified and LNA-modified probes. Mismatch discrimination in 69 mM Na^+ buffer is similar to mismatch discrimination in 1 M Na^+ (data not shown). Therefore, the same guidelines for probe design can be applied in magnesium buffers as well as sodium buffers of various concentrations. Interactions of Na^+ and Mg^{2+} ions with probe-target duplexes are not significantly mismatch specific. The mode of binding to nucleic acids is comparable for both ions and depends essentially on backbone negative charge density, i.e. distribution of phosphate groups (25). Since LNA residues do not introduce any significant charges, this result is not unexpected.

To further investigate effects of LNA modifications on counterion binding, perfectly matched duplexes from Sets 1DNA, 1T, 2T, 1ET, 1ES were melted in NaCl/phosphate buffers (19) at sodium ion concentrations of 69, 119, 220 and 621 mM Na^+ . Figure 5 plots dependence of melting temperatures on sodium concentrations. When the percentage of LNA-modified bases was increased from 0 to 47% in these sets, the duplex stability increased. The solid lines in Figure 5 were not obtained from fits to experimental data, rather they are calculated from published T_m salt correction (19) using melting temperatures in 1 M Na^+ as a reference,

$$\frac{1}{T_m(\text{Na}^+)} = \frac{1}{T_m(1\text{MNa}^+)} + (4.29f(\text{G}\bullet\text{C}) - 3.95) \times 10^{-5} \ln [\text{Na}^+] + 9.40 \times 10^{-6} \ln^2 [\text{Na}^+]. \quad 2$$

This T_m salt correction function was derived for native DNA–DNA duplexes and is observed to also apply to LNA–DNA duplexes, an important result that simplifies T_m predictions for LNA duplexes in various ionic environments.

Assuming a two-state melting transition, the number of sodium counterions released from the duplex, Δn , can be

estimated from the experimental T_m data (25),

$$\Delta n = \frac{d\left(\frac{1}{T_m}\right)}{d \ln [\text{Na}^+]} \frac{\Delta H^\circ}{\alpha R}, \quad 3$$

where R is the ideal gas constant, and α is the correction term for the sodium ion activity coefficient and is about 0.92 at 100 mM Na^+ . Transition enthalpies, ΔH° , were estimated from fits of melting profiles to two-state model (26). The values of Δn calculated at 100 mM Na^+ for T•A duplexes are 3.5, 3.5, 3.6, 3.6 for Sets 1DNA, 1T, 1ET, 1ES, respectively. The Δn can be predicted from the published empirical relationship (19), which was obtained from a large set of 92 duplex DNAs,

$$\Delta n = 2(N_{\text{bp}} - 1)[0.17 - 0.044f(\text{G}\bullet\text{C})]. \quad 4$$

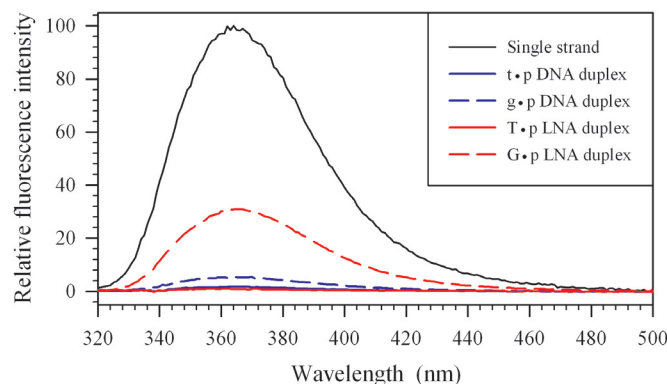
The predicted value of 4.1 is in reasonable agreement with experimentally measured Δn values. The results show that LNA modifications do not change amount of counterions that are released when a duplex is melted.

Fluorescence of 2-aminopurine reveals changes in stacking interactions introduced by LNA modifications

Stacking interactions within the mismatch site were investigated using a 2-aminopurine base (p). Fluorescence of this base is sensitive to local environment and is quenched when the base is stacked to neighboring bases (27,28). Magnitude of the fluorescence signal correlates with the relative extent of stacking interactions in duplex oligomers. The 2-aminopurine base is complementary to thymine (29,30) and forms a hydrogen bonded base pair that has similar dimensions to Watson–Crick T•A base pairs.

DNA oligomer target 5′-gcgaggpggctt-3′ with a central 2-aminopurine base was hybridized to four different strands (Figure 6). Fluorescence of the target oligomer and the four duplexes was determined. Figure 6 shows the results of UV melting and fluorescence measurements. Standard UV melting experiments demonstrate that a triplet of LNAs at the mismatch site stabilizes the duplex and increases mismatch discrimination in comparison with unmodified DNAs. Specifically, T_m difference between matched (t•p) and mismatched (g•p) duplexes is 9.5°C for native DNA oligomers and 15.9°C for the LNA probes. Fluorescence of the single stranded target (black line) was much larger than fluorescence of any of the duplexes, indicating that the 2-aminopurine base remains substantially stacked in all of the mismatched base pair combinations. The fluorescence spectra showed greater quenching for the DNA t•p base pair and the greatest quenching (and therefore base stacking) was observed for the LNA T•p base pair. Introduction of a mismatch increases fluorescence and decreases stacking interactions. The LNA-modified duplex with G•p mismatch resulted in more fluorescence than the native g•p DNA duplex, which suggests that the improved mismatch discrimination seen with LNA bases originates from subtle changes of base stacking interactions. We propose that LNA modifications may enhance stacking interactions of matched base pairs and suppress stacking interactions of mismatch base pairs relative to base stacking in native DNA duplexes.

LNA modification introduces an *O*-methylene group, which ‘points’ into the minor groove (15,24). A stabilizing water



Name	Sequence (5' to 3')	T_m ($^{\circ}\text{C}$)
t.p DNA	a a g c c t c c t c g c	58.3
g.p DNA	a a g c c g c c t c g c	48.8
T.p LNA	a a g c c <u>T</u> c c t c g c	73.2
G.p LNA	a a g c c <u>G</u> c c t c g c	57.3

Figure 6. Fluorescence emission spectra of target oligomer, 5'-gcgagppgctt-3', with single 2-aminopurine (p) reveal the magnitude of stacking interactions. Duplexes containing a H-bonded t

+ buffer showed that LNA triplets increased mismatch discrimination for these sequences.

bridge between 2' and 3' oxygen atoms of the ribose moiety has been proposed for various 2'-O-modifications (31). It is likely that the LNA modification alters the hydration pattern in the DNA minor groove. Stable water structure in the vicinity of the O-methylene bridge might contribute to increased duplex stability. Perturbations of hydration patterns and a direct role for hydration energetics in discriminatory effects of LNAs have yet to be proven.

Mismatch discrimination is dependent on oligomer length and mismatch position

Tables 1 and 2 show the effects that various mismatches have on T_m and $\Delta_{\text{md}}T_m$ values for 15 and 23 bp oligomers. Values vary with oligomer length and the impact of a single mismatch on overall duplex stability decreases with length. Figure 7 illustrates the dependence of mismatch discrimination on duplex length. Duplexes with a single g•t or a•c mismatch were examined, because these mismatches represent extremes, being the least and the most destabilizing mismatches, respectively. We assumed that average transition enthalpy and entropy of perfectly matched duplexes containing 50% g•c base pairs follow these relationships (22),

$$\Delta H = -8230(N_{\text{bp}} - 1) + 2400 \text{ cal/mol} \quad 5$$

$$\Delta S = -22.1(N_{\text{bp}} - 1) + 1.3 \text{ cal}/(\text{mol}\cdot\text{K}) \quad 6$$

which correspond to average values derived from the nearest-neighbor parameters (32). Figure 7 shows that a single a•c mismatch always destabilizes the duplex more than a single g•t mismatch. Discrimination is strongly dependent on length

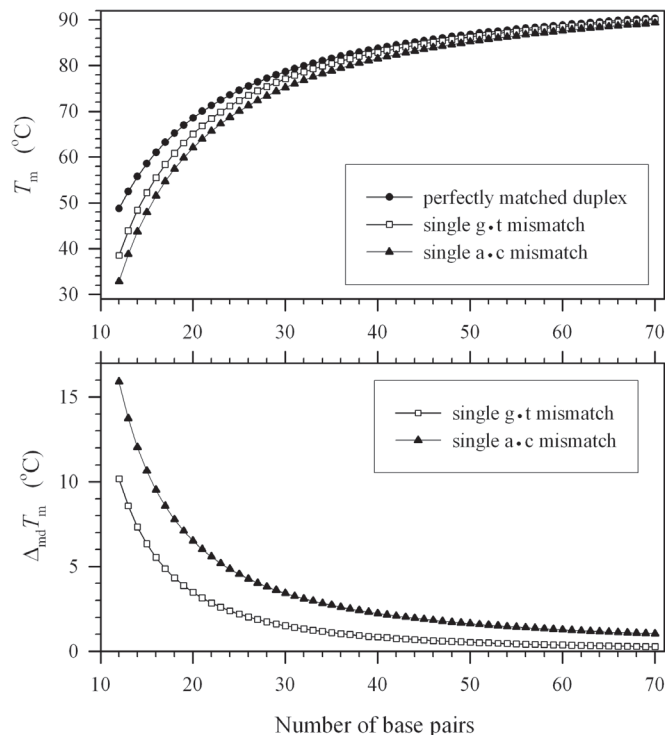


Figure 7. Dependence of melting temperature and mismatch discrimination on oligomer length. Average melting temperatures were calculated for 50% g•c DNA duplex oligomers that did not contain any mismatched base pairs as well as oligomers with single g•t or a•c mismatches. Predictions assumed total single strand concentration of 400 nM in 1 M Na⁺ buffer.

for short oligomers and decreases as oligomer length is increased. Decrease of discriminatory power is very significant in the 10–25 bp length range. For example, the $\Delta_{\text{md}}T_m$ values for an a•c mismatch decrease from 16 to 4.5 $^{\circ}\text{C}$ as duplex length is increased from 12 to 25 bp. Accurate predictions of mismatch discrimination can be calculated from the nearest-neighbor model for the particular DNA sequence and mismatch studied (32). Since LNA substitutions increase duplex stability, LNA probes can be designed to be shorter than unmodified DNA probes at the same site, allowing use of shorter probes and further enhancing mismatch discrimination.

Location of a mismatch within the probe sequence is also important. UV melting experiments have demonstrated (32) that terminal and penultimate mismatches have much smaller discriminating power than internal mismatches. Similar results were obtained using DNA microarrays (33). Duplexes with mismatches in the third to fifth position from the 5' end were reported to have significantly lower T_{ds} than those with mismatches in the first or second position. If the mismatch site is located 3 or more base pairs from the ends of oligomer, nearest-neighbor model assumes that energetic effects of the mismatch are position independent. To check these assumptions, we prepared a series of duplexes that contained a G•A mismatch at different positions and compared these to a perfectly matched T•A pair. Mismatch sites occurred either 3 bp from the 5' end, 3 bp from the 3' end or in the center of the duplex. In all cases, irrespective of location, the mismatches were embedded within the same sequence

Table 3. Comparison of melting temperatures and mismatch discrimination for duplexes containing the same G•A mismatch either in the middle versus near the ends of the sequence

Sequence (5'–3') ^a	LNA T_m (°C) ^c	ΔT_m (°C) ^d	DNA ^b T_m (°C) ^c	ΔT_m (°C) ^d
Perfectly matched T•A duplexes				
tATGaccttatgaggatgatga	69.7	—	65.2	—
tatgaccttATGaggatgatga	71.4	—	65.2	—
tatgaccttatgaggatATGa	70.6	—	65.2	—
Mismatched G•A duplexes				
tAGGaccttatgaggatgatga	64.9	4.8	63.4	1.8
tatgaccttAGGaggatgatga	65.5	5.9	61.7	3.5
tatgaccttatgaggatAGGa	64.7	5.9	63.0	2.2

^aComplementary DNA target was 5'-tcatatcctcataaggtcata-3'. Mismatch sites are underlined, DNA bases are lowercase, LNA bases are uppercase.

^bLNA bases were replaced with native DNA bases for this set of duplexes.

^cMelting temperatures were determined in 1 M Na⁺ buffer and $C_T = 2 \mu\text{M}$.

^d ΔT_m is calculated from the difference between perfectly matched and mismatched duplexes.

context, -TAXGA-, so any sequence-dependent effects on duplex stability were minimized. UV melting experiments were conducted and T_m values were determined for each duplex; results are presented in Table 3. As expected, LNA triplets improved mismatch discrimination by $\sim 3.0^\circ\text{C}$. The position of the mismatched base pair had a small influence on mismatch discrimination. The mismatch located in the center of the sequence showed slightly improved discrimination compared to mismatches located closer to the ends of the duplex. Interestingly, this position preference was more pronounced for DNA probes than for LNA probes. Therefore, whenever possible positioning the mismatch site close to the center of the probe-target duplex is preferred to achieve maximum discriminatory power. Letowski *et al.* (34) observed similar positional effects using microarrays. They reported that 50 bp long probes showed better specificity when five distributed mismatches were located in the middle of the probe. Probes with mismatches located at either end of the molecule were less discriminating.

Stability predictions for LNA–DNA duplexes

Stability of perfectly matched LNA–DNA duplexes can be estimated from published nearest-neighbor parameters (32,35) for mixed sequences containing LNA bases interspersed among DNA bases. LNA parameters (35) predict energetic differences between LNA-modified and unmodified DNA duplexes. We therefore tested if the parameters were accurate in predicting changes of melting temperatures due to LNA substitutions. Experimental $T_m(\text{LNA}) - T_m(\text{DNA})$ values of perfectly matched duplexes are presented in Table 4 and are compared with values calculated using the 'unified' (32) and LNA (35) parameter sets. The T_m increases caused by LNA modifications were predicted for a combined set of all LNA probes with an average error of $\pm 0.7^\circ\text{C}$, which is within the experimental error. However, predictions of T_m changes were less accurate for the 1ET Set ($\pm 1.6^\circ\text{C}$) and even less accurate for the 1ES Set ($\pm 2.3^\circ\text{C}$), where half of the nucleotides were LNA-modified. The published LNA thermodynamic parameters (35) give a more accurate

prediction of duplex stabilization when LNA residues are present in low densities.

Since nearest-neighbor parameters for LNA mismatches are unknown, thermodynamic stability of mismatched LNA–DNA duplexes cannot be accurately predicted. Melting data presented in Tables 1 and 2 are not sufficient to derive these parameters, but will help in future investigations. Here, two base sequences were employed and the relative positions of LNA modifications were varied. To obtain nearest-neighbor parameters, it will be necessary to study a much larger set of sequences with the same kind of modification pattern. This study suggests that it would be most useful to determine parameters that can predict thermodynamics for triplets of LNA residues. Accurate stability predictions would make the process of designing LNA probes more reliable.

New design guidelines and biological applications

Although LNA synthesis methods have improved in recent years, LNA phosphoramidite monomers still exhibit lower coupling efficiency than DNA monomers. In particular, coupling efficiency of guanine LNA monomer is lower than the other LNA residues. It is better to avoid synthesis of long stretches of consecutive LNA bases because such sequences give lower synthesis yields, lower purity, and may therefore require PAGE or HPLC purifications.

Any probe candidates, including LNA probes, should be examined for their tendency to fold into hairpins, homodimers and other undesirable secondary structures. Stability of such secondary structures need to be minimized to prevent competition with desired probe-target duplex formation. Thermodynamic experiments have demonstrated (36) that LNA–LNA base pairs are more stable than LNA–DNA base pairs, which are more stable than DNA–DNA base pairs. It is therefore likely that problems arising from secondary structures will be more significant when LNA residues are present.

Combining our experimental observations with previous suggestions (1,2,5,11), we propose the following rules to maximize single mismatch discrimination using LNA probes:

- (i) Place a triplet of LNA modifications with the central base of the triplet at the mismatch site, unless the probe contains the guanine base of a G•T mismatch.
- (ii) LNA modification of the guanine nucleotide or either of its nearest-neighbor bases should be avoided in a G•T mismatch site.
- (iii) Shorter probes improve mismatch discrimination.
- (iv) For best synthesis quality, it is preferable to avoid consecutive sequence of more than four LNA residues.
- (v) Better discrimination seems to be achieved when the position of the mismatch site is close to the center of the probe. Discrimination is significantly decreased if mismatches are located at the first or second base from either end of the duplex.
- (vi) If a mismatch type and location are unknown, the best approach is to modify all adenine nucleotides until the optimal T_m is achieved. The rest of the rules should be taken into account.
- (vii) Probes should not fold into stable, undesired self-complementary secondary structures or form self-dimers, especially when these structures contain LNA–LNA base pairs.

Table 4. Comparison of predicted and experimentally measured melting temperatures ($^{\circ}\text{C}$) for perfectly matched DNA and LNA probe-target duplexes in 1 M Na^+ buffer

Set	X•Y base pair	T_m of DNA–DNA duplexes		T_m of LNA–DNA duplexes		$T_m(\text{LNA}) - T_m(\text{DNA})$ ($^{\circ}\text{C}$) ^c		Error ^d
		Experimental ^a	Predicted ^b	Experimental ^a	Predicted ^b	Experimental	Predicted	
1M	A•T	59.4	61.9	60.9	63.2	1.5	1.3	-0.2
	T•A	60.5	62.7	62.9	65.0	2.4	2.3	-0.1
	C•G	61.8	65.3	65.6	68.7	3.8	3.4	-0.4
	G•C	63.9	66.5	65.1	68.2	1.2	1.7	0.5
1NN	A•T	59.4	61.9	65.7	69.3	6.3	7.4	1.1
	T•A	60.5	62.7	65.8	68.4	5.3	5.7	0.4
	C•G	61.8	65.3	68.1	71.7	6.3	6.4	0.1
	G•C	63.9	66.5	69.5	73.1	5.6	6.6	1.0
1NNN	A•T	59.4	61.9	63.1	65.3	3.7	3.4	-0.3
	T•A	60.5	62.7	64.1	66.0	3.6	3.3	-0.3
	C•G	61.8	65.3	65.6	68.7	3.8	3.4	-0.4
	G•C	63.9	66.5	66.9	69.9	3.0	3.4	0.4
1ES	A•T	59.4	61.9	79.7	78.7	20.3	16.8	-3.5
	T•A	60.5	62.7	79.0	80.7	18.5	18.0	-0.5
	C•G	61.8	65.3	83.3	84.4	21.5	19.1	-2.4
	G•C	63.9	66.5	83.5	83.5	19.6	17.0	-2.6
1ET	A•T	59.4	61.9	72.8	75.8	13.4	13.9	0.5
	T•A	60.5	62.7	73.3	77.9	12.8	15.2	2.4
	C•G	61.8	65.3	76.5	81.6	14.7	16.3	1.6
	G•C	63.9	66.5	76.4	80.8	12.5	14.3	1.8
1A	A•T	59.4	61.9	63.6	66.1	4.2	4.2	0.0
	T•A	60.5	62.7	64.4	66.2	3.9	3.5	-0.4
	C•G	61.8	65.3	65.6	69.1	3.8	3.8	0.0
	G•C	63.9	66.5	67.4	70.3	3.5	3.8	0.3
1B	A•T	59.4	61.9	62.0	64.8	2.6	2.9	0.3
	T•A	60.5	62.7	62.5	64.7	2.0	2.0	0.0
	C•G	61.8	65.3	64.3	67.7	2.5	2.4	-0.1
	G•C	63.9	66.5	66.2	69.3	2.3	2.8	0.5
2M	A•T	75.8	77.2	77.3	78.5	1.5	1.3	-0.2
	T•A	74.1	75.3	77.3	78.5	3.2	3.2	0.0
	C•G	77.7	80.7	81.8	85.1	4.1	4.4	0.3
	G•C	78.8	80.3	80.7	82.6	1.9	2.3	0.4
2NN	A•T	75.8	77.2	83.8	84.8	8.0	7.6	-0.4
	T•A	74.1	75.3	83.5	83.4	9.4	8.1	-1.3
	C•G	77.7	80.7	87.1	88.7	9.4	8.0	-1.4
	G•C	78.8	80.3	87.3	88.6	8.5	8.3	-0.2

^aExperimental melting temperatures at C_t of 2 μM .^bPredicted melting temperatures using unified (32) and LNA (35) nearest-neighbor parameters.^cIncrease of T_m due to LNA modifications.^dError of predicted T_m hike.

Rules (iii) and (iv) should be balanced depending on the precise needs of specific applications. Fully modified short LNA probes (6–8 bp) have been used successfully in SNP detection (13,14) experiments. They are less suitable for some high-throughput screenings where each experiment cannot be thoroughly optimized, and varying synthesis yields as well as extensive purifications are incompatible. Longer LNAs (>14 bp) may possess too high affinity for complementary strands when they are fully modified. Additional experimentation is needed to obtain more insight about advantages and disadvantages of short fully LNA-modified probes versus chimeric LNA/DNA probes. The guidelines presented herein are available in the IDT SciTools OligoAnalyzer calculator (freely accessible at www.idtdna.com), which can predict stability of LNA–DNA duplexes and assist with probe design.

DNA microarrays are a powerful technology for parallel high-throughput analysis of gene expression and genotyping. Since a single set of hybridization and wash conditions (temperature, ionic strength) is typically employed, design of microarrays that can reliably distinguish single base variations

on a genomic scale represents a real challenge. LNAs can be used to improve microarray probes because they allow use of shorter and therefore better discriminating sequences. Ideally, at the hybridization temperature employed, perfectly matched probe-target duplexes should be stable and mismatched duplexes should be unstable. In this setting, the increased discriminatory power and specificity of LNA probes may enable more definitive results. The stability of duplexes increases with increasing G•C content. LNA bases can be selectively incorporated to fine-tune and control thermal stability, serving to normalize the T_m of large numbers of heterogeneous probe sequences. Probes with low $f(\text{G}\bullet\text{C})$ could contain more LNA residues than probes with high $f(\text{G}\bullet\text{C})$. The number of LNA residues and lengths of probes could be adjusted, so that various probe-target duplexes achieve similar stability.

LNA probes have been successfully used in quantitative real-time PCR applications (1–5). Improved sensitivity and specificity have been observed in genotyping experiments and SNP polymorphism analysis. The number of LNA

modifications can be adjusted to optimize melting temperatures of probe-target duplexes. Primers are usually designed to have very similar melting temperatures in the range of 55–60°C and T_m of the probe is recommended to be about 7–10°C higher (3). LNA modifications allow use of shorter fluorescent probes, improve mismatch discrimination and increase the window of annealing temperatures in which accurate genotyping results can be achieved (1). Various formats of detection are possible. Besides dual-labeled probes, unlabeled LNA probes with LCGreen or SYBR Green dyes can be used to improve sensitivity of melting curve analysis (4).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. The authors are employed by Integrated DNA Technologies, Inc., (IDT) which has filed, or may have filed, at least one patent application on the materials or methods described in the preceding paper, and which offers oligonucleotides for sale similar to the oligonucleotides described in this paper. IDT is not, however, a publicly traded company, and none of the authors own any equity in IDT.

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