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Position-dependent effects of locked nucleic acid (LNA) on DNA sequencing and PCR primers

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ABSTRACT

Genomes are becoming heavily annotated with important features. Analysis of these features often employs oligonucleotides that hybridize at defined locations. When the defined location lies in a poor sequence context, traditional design strategies may fail. Locked Nucleic Acid (LNA) can enhance oligonucleotide affinity and specificity. Though LNA has been used in many applications, formal design rules are still being defined. To further this effort we have investigated the effect of LNA on the performance of sequencing and PCR primers in AT-rich regions, where short primers yield poor sequencing reads or PCR yields. LNA was used in three positional patterns: near the 5′ end (LNA-5′), near the 3′ end (LNA-3′) and distributed throughout (LNA-Even). Quantitative measures of sequencing read length (Phred Q30 count) and real-time PCR signal (cycle threshold, C_T) were characterized using two-way ANOVA. LNA-5′ increased the average Phred Q30 score by 60% and it was never observed to decrease performance. LNA-5′ generated cycle thresholds in quantitative PCR that were comparable to high-yielding conventional primers. In contrast, LNA-3′ and LNA-Even did not improve read lengths or C_T. ANOVA demonstrated the statistical significance of these results and identified significant interaction between the positional design rule and primer sequence.

INTRODUCTION

Oligonucleotide-based technologies enable better understanding of cellular function and inherited disease. These technologies rely on nucleic acid hybridization for their sensitivity and specificity. However, for a growing number of applications, unmodified oligonucleotides (comprising solely DNA or RNA nucleotides) sometimes yield unacceptable results. Hybridization assays can be difficult to design because important biological features such as SNPs, CpGs, exons, splice sites and protein binding regions are sometimes located in sequence contexts that are poor for design. For example, simple repetitive sequence is poor for primer design, and SNPs may be embedded in short tandem repeats. Features may lie in poor sequence context simply due to the variety of sequence contexts in the genome. Assays may also fail if a method requires hybridization at a defined position relative to the feature of interest. For example, the SNP single base extension method (1) requires the 3′ terminal terminal base of a primer to be immediately adjacent to the SNP. Methylation Specific PCR similarly requires the bisulfite-treated C of the CpG of interest to be at or near the 3′ end of the primer (2).

Nucleic acid analogues have a range of hybridization properties that allows modified oligonucleotides to be used successfully in locations where unmodified oligonucleotides fail. Nucleic acid base modifications such as inosine and 7-deazaguanosine reduce melting temperature and have been used to improve primers in GC-rich templates (3). One of the most promising analogues used to raise melting temperature is Locked Nucleic Acid (LNA). LNA is a bicyclic ribose derivative with a bridging methylene group between O-2′ and C-4′. LNA provides the largest known increase in thermal stability of any modified DNA duplex (4), because it reduces the unfavorable entropy of duplex formation and may improve base stacking. LNA also has greater mismatch sensitivity than DNA (5,6). LNA is available for all four bases, so it is easily placed at any position in an oligonucleotide.

LNA has been used in many applications. SNP applications include: TaqMan® (7); Molecular Beacons (6); fluorescence polarization (5); and immobilization (8) probes. A partial list of other applications includes PCR primers (9), allele-specific PCR (10), real-time PCR probes (11,12), antisense

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oligonucleotides (13), siLNA duplexes (14,15), single molecule miRNA detection (16), miRNA probes (17), decoy oligonucleotides (18), microarray probes (19), aptamers (20) and sequence detection using piezo-resistive cantilevers (21).

Effective design of LNA oligonucleotides requires accurate thermodynamic parameters (14). Our group recently determined sequence-dependent parameters for single, internal LNA incorporation into DNA duplexes (22). The effects of LNA on duplex stability are highly sequence-dependent. Moreover, the stabilization may be either entropic or enthalpic, depending on sequence context (22). These parameters provide precise estimates of duplex melting temperature. Their incorporation into Celadon's ProbeITy™ oligonucleotide assay design software enables the design of LNA primers and probes.

Effective design also requires positional rules (14). Latorra *et al*. (9) provided the first systematic study of the effect of LNA position and PCR conditions on LNA primer efficiency. They modified a single primer sequence with 11 LNA patterns and used the intensity of final product bands as the measure of priming efficiency. They found that LNA should be spaced evenly across the primer and that no more than three LNA should be used. This research design does, however, confound LNA number with its pattern of incorporation and real-time PCR would provide a more accurate and precise estimate of any LNA effects. Exiqon, the intellectual-property holder of LNA, does not make positional recommendations at this time on their web site.

The goal of the present study is to generate LNA positional design rules for sequencing and PCR primers. The effects of LNA on the two methods are expected to be similar. Our research design was to first identify a number of short DNA primers that generated poor sequencing reads. We then modified each primer with three different patterns of LNA incorporation (LNA-5′, LNA-Even and LNA-3′) with the goal of testing which patterns improve performance. Performance is assessed quantitatively using sequencing base call quality scores and PCR cycle thresholds. ANOVA enabled us to assess the magnitude of the effects of different LNA incorporation patterns and it enabled for the first time the statistical testing of interaction between primer sequence and LNA pattern. Significant interaction would mean that a particular pattern of LNA incorporation improved the performance of some primers, but degraded the performance of others.

We found that the LNA-5′ incorporation pattern significantly improved both sequencing and PCR primers, but LNA-3′ and LNA-Even did not. These results probably arise because LNA near the 5′ end enhances stability without increasing mispriming, while LNA near the 3′ end stabilizes mispriming, although our results cannot exclude the possibility of direct LNA effects on polymerase extension. The ANOVA research design also provided objective evidence for interaction, meaning that the effect of LNA incorporation depends on primer sequence.

MATERIALS AND METHODS

Template and primer design and synthesis

The DNA sequencing templates were PCR amplicons from the human membrane cofactor protein (MCP) gene (AL035209) and the human ataxia telangiectasia mutated (ATM) gene (AP001925). All PCR and sequencing primers were designed using ProbeITy™ oligonucleotide assay design software (Celadon Laboratories, Inc., Hyattsville, MD; www.celadonlabs.com). Unmodified DNA sequencing primers were shortened progressively to derive low-T_M, poor-quality sequencing primers, as described in Results. Placement of LNA into the primers was guided by prototype software that implemented a database of thermodynamic parameters for single LNA incorporations (22). DNA and LNA-enhanced primers (all from IDT, Coralville, IA) were suspended in 1× TE at ~100 μM and concentration was measured using absorbance.

PCR to generate sequencing templates was performed in 10–50 μl reactions using 200 nM of each PCR primer, 165 ng genomic DNA (Coriell, Camden, NJ) and 1.25 U AccuPrime *Taq* DNA polymerase with AccuPrime buffer II, which includes dNTPs (Invitrogen, Carlsbad, CA). Thermal cycling began with denaturation at 94.0°C for 2 min followed by 30 cycles of 94.0°C for 30s, 60.0°C for 30s and 68.0°C for 80s. PCR product was visualized on 1.5% agarose gels with ethidium bromide. PCR products were purified using Centricon-100 micro-concentrators (Millipore, Bedford, MA).

DNA sequencing reactions

Sequencing reactions on purified PCR product templates were performed by the DNA Sequencing Facility at the Center for Biosystems Research (CBR), University of Maryland Biotechnology Institute. The ABI Big Dye Terminator DNA Sequencing kit (V. 3.1) and ABI thermal cyclers were used, with an annealing temperature of 50°C. Sequencing traces were produced on the ABI 3730 96-channel, 50 cm array DNA Analyzer and base quality scores were generated using the Phred algorithm included with the ABI 3730 software (23,24). To ensure that all samples were properly tracked, every trace was aligned with its intended target sequence using Sequencher (GeneCodes, Inc., Ann Arbor, MI). After an array upgrade early in the project, replicates performed in the same sequencing batch and control samples that were run on different days exhibited negligible variation.

Quantitative assessment of sequence read quality

For hypothesis testing for the ANOVA research design, Phred counts were selected as a quantitative measure of sequencing read quality. Phred is an algorithm (23,24) that generates sequence reads by calling bases in the trace and assigning an estimated error probability P and a quality score Q to each call. The values are related by $Q = -10[\log_{10}(P)]$. A Phred Q score of 20 equates to a 99.0% probability that the base-call is correct and a Phred Q score of 30 is a 99.9% probability. Phred $Q = 20$ is the threshold quality standard for genome sequencing projects [Ref. (25) and http://www.genome.gov/10000923].

Our quantitative measures of sequencing quality are the numbers of bases that were assigned Phred Q scores $\geqslant$20 or $\geqslant$30 in the first 1000 bases of DNA sequence. We define these numbers of bases as the Phred Q20 and Q30 counts. For sequencing where the quality remains high until near the end of the usable data, the Phred Q30 score is essentially

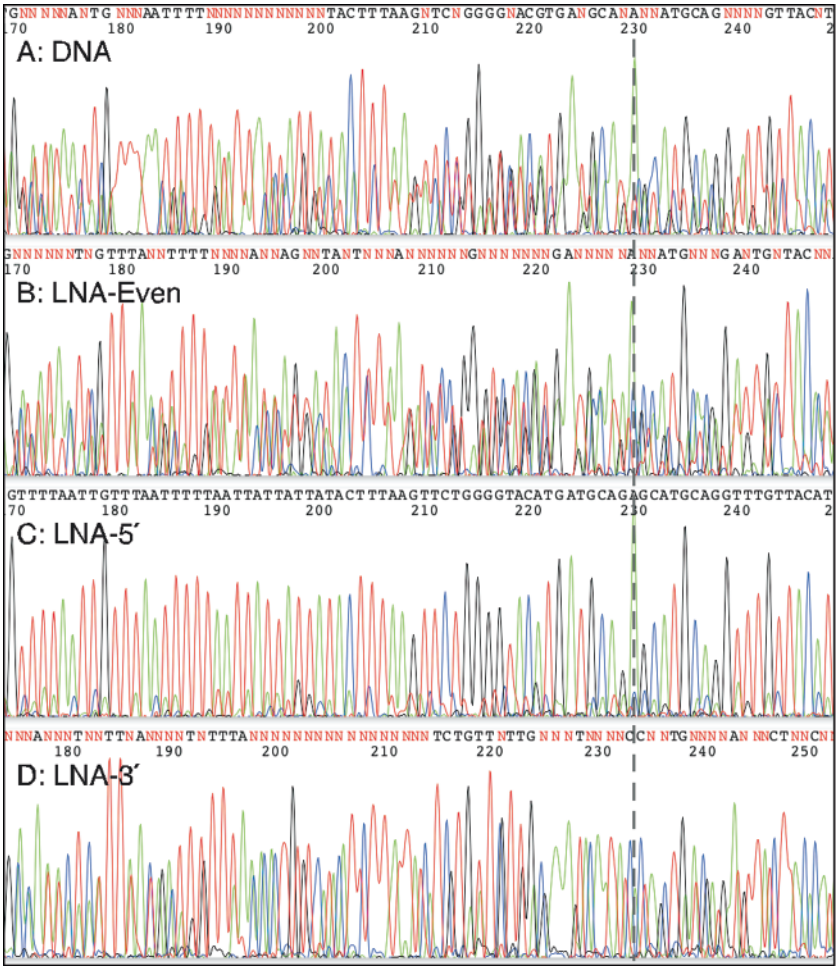

Figure 1. Representative effects of LNA incorporation pattern on sequencing electropherograms. Portions of electropherograms generated from ATM2 primers with the indicated LNA substitution patterns are shown. The same AT-rich ATM PCR product was the template for all four reactions and all reactions were performed in the same plate. The primers used are as follows: (**A**) unmodified ATM2; (**B**) ATM2-LNA-Even; (**C**) ATM2-LNA-5′; (**D**), ATM2-LNA-3′. The panels are aligned at A230 as indicated by the dashed line.

the read length. In our data, Phred Q20 and Q30 counts were highly correlated ($r^2 = 0.985$), so we generally report only the Q30 counts. We observed a good qualitative correlation between high Phred Q20 and Q30 counts and subjectively robust sequencing reads, those with strong fluorescent peaks and little or no overlap. Noisy electropherograms with overlapping fluorescent peaks generated low Phred Q20 and Q30 counts. (See the sample electropherograms in Figure 1.)

Real-time PCR with SYBR green detection

Real-time PCR was performed in 96-well plates with optical caps using a GeneAmp 5700 (Applied Biosystems). Reaction volume was 50 µl, including a SYBR Green PCR Master Mix that included enzyme, Mg^{2+} and dNTPs (ABI, Foster City, CA), 100 nM each PCR primer and 0.5 ng/µl genomic DNA. A hot start of 2 min at 50°C and 10 min at 95.0°C was followed by 40 cycles of 95.0°C for 15 s and 60.0°C for 60 s. The identities of the PCR products were confirmed using restriction analysis. For comparison of different forward primers, master mixes were used that included all the other components, including the appropriate reverse primer. The cycle threshold (C_T), defined as the thermal cycle at which the signal intensity surpassed a value of 0.1, was used as the quantitative measure of real-time PCR quality.

A dissociation curve from 60.0 to 92.0°C was performed following each PCR run. All primer sets used in this study exhibited a single peak between 70.0 and 75.0°C. This relatively low amplicon melting temperature is due to their AT-rich composition. Control reaction chambers that contained the master mix with only the reverse primer did not generate a peak in the dissociation curve, indicating that the reverse primer alone did not generate genomic product or signal due to primer-dimers. Control standard PCR showed no product in the absence of template.

Prediction and measurement of primer melting temperatures

To predict the melting temperature of the DNA primers under PCR conditions we assumed 50 mM KCl, 1.8 mM $MgCl_2$, 1 mM dNTPs and 200 nM total primer concentration. The unified ΔH° and ΔS° nearest-neighbor parameters of SantaLucia (26) and a standard salt correction formula (27)

were used. To predict melting temperatures under thermal melt curve conditions we assumed 1 M NaCl and 2 μM total primer concentration, typically giving T_M values 10–15°C higher. UV absorbance versus temperature curves were obtained and analyzed as previously described (22).

To predict melting temperature for the DNA–LNA primers we added the LNA sequence-dependent ΔΔH° and ΔΔS° parameters from Ref. 22 to the ΔH° and ΔS° values obtained as above. For 5′-terminal LNA substitutions, we treated the terminal LNA the same as an internal modification. This analysis ignores possible differences between internal and terminal positions. We also ignore possible cooperative effects between multiple LNAs within a sequence. This assumption that the thermodynamic effects of each LNA are additive and independent is unlikely to be correct. The effect of additional LNA on T_M plateaus, so subsequent incorporations have smaller effects than the initial one (28).

ANOVA

The research design was a two-factor, mixed-model ANOVA with replication. The two factors were LNA position and primer sequence. The mixed-model aspect derives from the fact that chemistry (LNA position) is a treatment effect fixed by us, while there was no such experimental manipulation of primer sequences after their initial selection.

Calculations were performed using the 'ANOVA-Two-Factor with Replication' Analysis ToolPak add-in of Microsoft Excel. Certain *F*-tests were performed manually, because Excel computes *F*-statistics by dividing all mean squares by the error mean square regardless of the statistical significance of the interaction. However, when the interaction is significant, as it was in our data, the correct *F*-statistic computation for a mixed model is to divide the fixed treatment effect mean square (in our case, LNA pattern) by the interaction mean square (see Ref. 29, pp. 337–338). ANOVA assumes that data are normally distributed. Our data provide insufficient power to reject the normal distribution regardless of its true distribution. However, variation in the Q30 count is likely to have a normal distribution.

The first ANOVA procedure analyzed differences in the mean Q30 count among all LNA patterns. This analysis was globally informative of differences among means, but it was not informative of which mean or groups of means were statistically different from other means or groups of means. To analyze these differences required comparisons, which are indicated only when the global analysis is significant. Possible comparisons among LNA patterns include six pair-wise comparisons and there were also comparisons among groups of means, such as the unmodified primer versus the three LNA patterns. The decision on which comparisons to perform was influenced by the available degrees of freedom, which in this case is three because there are four chemistry patterns.

Since our basic premise was that the LNA patterns would generate significantly different Q30 scores when compared to the unmodified primers, if the global analysis was found to be significant the obvious choice was to make pair-wise comparisons of mean Q30 score between each of the three LNA patterns and the unmodified primer. These three planned comparisons consume, but do not exceed, the available three degrees of freedom. *P*-value corrections for multiple comparisons were not performed because these are only necessary when the number of comparisons exceeds the degrees of freedom.

RESULTS

Experimental design

LNA incorporation is most likely to be necessary for improving the performance of short, low-T_M, AT-rich primers. Using six such primers, we sought to identify positions in sequencing primers at which LNA has a statistically significant effect on sequencing quality, either positive or negative. Our expectation was that at least one LNA pattern would lead to higher sequencing quality, because LNA increases primer melting temperature. Three LNAs were incorporated into each primer, because this was sufficient to elevate the predicted melting temperatures into the range that produced good quality sequencing reads. Previous worked showed that PCR failed when primers had more than three LNAs (7).

LNA was distributed across the test primers in three different patterns. The primer sequences were divided into three segments of equal length: 5′, middle and 3′. The 'LNA-5′' pattern had two LNAs in the 5′ section and one in the middle. The 'LNA-Even' pattern had one LNA in each section. The 'LNA-3′' pattern had one LNA in the middle and two in the 3′ section. This research design, using four primers with identical base sequences but differing in composition, isolates the effect of LNA. Sequencing reactions for each primer were performed in triplicate to generate within-primer variances. This approach allowed us to assess the amount of variation that was due to interaction between primer sequence and LNA pattern. We applied the same research design to PCR primers.

Selection and characterization of poor unmodified DNA sequencing primers

Primers were designed for two arbitrarily chosen realistic test cases, the AT-rich human disease genes (MCP/CD46) and ATM. MCP is a receptor for a wide variety of pathogens (29) and ATM is a central regulatory gene in the cellular response to DNA damage (30). We expect that the results obtained on these primers will generalize to all primers that have a melting temperature that is low with respect to the sequencing reaction temperature.

To obtain low-T_M primers that generated poor sequencing quality, for each of the two genes three sets of AT-rich primers that had predicted T_Ms of ~60°C were initially identified. One base at a time was removed from each primer's 5′ end until a primer with a predicted T_M of ~35°C was reached, which should be sufficiently low to ensure poor sequencing. From each of the six resulting sets of primers, about six primers were selected that sampled melting temperature every 3–4°C (sequences not shown). Triplicate sequencing reactions were performed for each selected primer. From each of the six sets, the highest-T_M primer that produced a poor sequencing read was chosen, resulting in six primers of Table 1, which ranged in predicted melting temperature (under sequencing conditions) from 34.0

to 40.0°C. These primers (ATM1, ATM2, ATM3, MCP1, MCP2 and MCP3) were modified with the LNA-5′, LNA-3′ and LNA-Even patterns.

After the LNA-modified primers were chosen and synthesized, the sequencing center upgraded the capillary array and sequencing procedures, which markedly improved the sequencing with the chosen MCP primers. We report results from the MCP1 primers from both before and after the upgrade in Tables 1 and 3. All the other results and all of the statistical analyses below for the ATM and MCP primers are for the upgraded array. The long and consistent post-upgrade MCP reads of Table 1 contrast with the short and variable ATM reads. This provided us with the opportunity to test the effects of LNA on primers of various read lengths and consistency.

T_M predictions for LNA-enhanced primers

To test melting temperature predictions for LNA-modified primers, we measured the T_M of each sequencing primer using standard melt conditions (22); see Table 2. Predictions for DNA primers were highly accurate: they deviated $<\pm 1.0$°C for five of the six primers. The T_M for ATM3 (CCAGAAAGCCA) was 2.6°C higher than predicted. This could be due to the short A-tract, which is known to generate aberrant melting behavior (31). For the LNA-substituted primers, the average difference between predicted and observed was also small, just +0.8°C. However, this average result masks a decrease in accuracy as compared to the all-DNA primers: the difference between observed and predicted ranged from −3.7° to +5.6° and 11 of the 17 primers had

Table 1. DNA sequencing primers used to test LNA incorporation patterns

Primer[a]	Sequence (5′–3′)	Length	GC (%)	Pred. T_M^c	Phred Q20[b]	Phred Q30[b]
MCP1	CTTAAATTTTCTTGAAT	17	18	39.4	892 ± 50	749 ± 89
MCP1[d]	CTTAAATTTTCTTGAAT	17	18	39.4	505 ± 227	281 ± 243
MCP2	AGCAATAAAGAAC	13	31	34.5	925 ± 8	799 ± 18
MCP3	TAAACACATCATC	13	31	33.6	895 ± 8	743 ± 7
ATM1	AGAGGAAGCATA	12	42	34.5	345 ± 153	123 ± 75
ATM2	CATATAAGTCAGAAT	15	27	36.1	158 ± 13	42 ± 3
ATM3	CCAGAAAGCCA	11	55	36.4	655 ± 159	387 ± 165

[a]The six low-T_M primers derive from the MCP and ATM genes.
[b]Phred Q20 and Phred Q30 counts are defined as the total number of bases out of the first 1000 bases in the sequencing electropherograms that have Phred scores of atleast 20 or 30 respectively. The average and standard deviation of three reactions is given.
[c]The T_M (in °C) is predicted for sequencing reaction conditions: 50 mM KCl, 50 mM NaCl, 1.8 mg Mg^{2+}, 100 nM total (primer), 1 mM total dNTPs.
[d]This row refers to earlier sequencing, during the sequence selection/design phase of the work, using the MCP1 primer under less optimal conditions.

Table 2. LNA-substituted sequencing primers

Primer	Sequence[a] (5′–3′)	T_M (°C) reaction Predicted[b]	T_M (°C) melt conditions Predicted	Measured	ΔT_M^c
MCP1-DNA	CTTAAATTTTCTTGAAT	39.4	53.5	54.9	−1.4
MCP1-LNA-3′	CTTAAATTTT+CT+TGA+AT	45.1	60.3	61.8	−1.5
MCP1-LNA-5′	+CT+TA+AATTTTCTTGAAT	43.9	59.0	57.5	1.5
MCP1-LNA-Even	CT+TAAAT+TTTCT+TGAAT	44.4	59.8	60.0	−0.2
MCP2-DNA	AGCAATAAAGAAC	34.5	49.5	49.8	−0.3
MCP2-LNA-3′	AGCAA+TAA+AG+AAC	42.0	58.4	60.9	−2.5
MCP2-LNA-5′	A+GC+AA+TAAAGAAC	40.6	56.6	55.8	0.8
MCP2-LNA-Even	A+GCAA+TAAAG+AAC	42.2	58.1	55.4	2.7
MCP3-DNA	TAAACACATCATC	33.6	48.5	50.4	−1.9
MCP3-LNA-3′	TAAACA+CAT+CA+TC	40.0	57.4	62.3	−4.9
MCP3-LNA-5′	+TA+AA+CACATCATC	38.8	55.3	55.2	0.1
MCP3-LNA-Even	TA+AACA+CATCA+TC	38.7	56.0	58.4	−2.4
ATM1-DNA	AGAGGAAGCATA	34.5	50.6	51.2	−0.6
ATM1-LNA-3′	AGAGGA+AG+CA+TA	44.3	62.1	62.7	−0.6
ATM1-LNA-5′	A+GA+GGA+AGCATA	45.6	62.7	64.0	−1.3
ATM1-LNA-Even	A+GAGG+AAGCA+TA	42.7	60.7	57.8	2.9
ATM2-DNA	CATATAAGTCAGAAT	36.1	50.6	51.2	−0.6
ATM2-LNA-3′	CATATAAG+TC+AGA+AT	40.8	56.8	58.6	−1.8
ATM2-LNA-5′	C+AT+ATA+AGTCAGAAT	38.9	55.1	57.4	−2.3
ATM2-LNA-Even	CAT+ATA+AGTC+AGAAT	40.5	56.5	58.6	−2.1
ATM3-DNA	CCAGAAAGCCA	36.4	52.5	50.9	1.6
ATM3-LNA-3′	CCAG+AAA+GC+CA	44.6	62.2	63.5	−1.3
ATM3-LNA-5′	+CC+AG+AAAGCCA	42.4	60.3	58.8	1.5
ATM3-LNA-Even	C+CAGA+AAGC+CA	42.7	61.5	59.6	1.9

[a]LNA positions are underlined and preceded by + signs.
[b]The T_M is predicted for the sequencing reaction conditions specified in the legend for Table 1
[c]The ΔT_M is the predicted minus observed under melt conditions.

differences >±1.0°. This confirms that the single-LNA substitution results from (22) must be extended in order to describe multiple incorporations.

The LNA-5′ incorporation pattern dramatically improves DNA sequencing

LNA incorporation can improve primer performance substantially, and the effect is markedly dependent on its pattern of incorporation (Table 3 and Figures 1 and 2). The electropherograms of Figure 1 show that the primary cause of low Phred Q30 and Q20 counts for the poorly performing ATM primers was multiple sequence ladders. Multiple ladders arise from mispriming, so the observed effects cannot be due to inhibition of polymerase extension by LNA; although LNA may well have some direct effect on the polymerase,

Table 3. Phred Q30 counts for sequencing with unmodified and LNA-substituted primers

Primer	LNA incorporation pattern			
	LNA-5′	No LNA	LNA-3′	LNA-Even
MCP1[a]	781 ± 2	281 ± 243	n.d.	54 ± 5
MCP1	814 ± 12	749 ± 89	469 ± 155	603 ± 60
MCP2	838 ± 13	799 ± 18	834 ± 9	832 ± 13
MCP3	799 ± 20	743 ± 7	756 ± 28	538 ± 61
Average	817 ± 22	764 ± 53	686 ± 184	658 ± 140
ATM1	621 ± 153	123 ± 75	109 ± 3	101 ± 18
ATM2	720 ± 77	42 ± 3	90 ± 13	52 ± 14
ATM3	757 ± 63	387 ± 165	150 ± 97	94 ± 14
Average	699 ± 110	184 ± 181	116 ± 56	82 ± 26
Overall	758 ± 98	474 ± 325	401 ± 321	370 ± 312

The average and standard deviation of the Phred Q30 Counts are given from three sequencing runs. See Table 2 for primer sequences.
[a]The data in this row are from earlier sequencing reactions done under non-optimal conditions. Two sequencing runs were performed for each LNA-modified primer. These results are not included in the averages below.
n.d., not done.

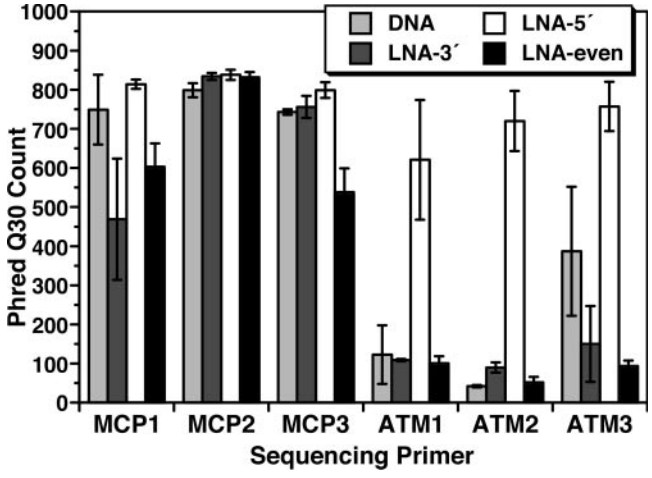

Figure 2. The effects of LNA incorporation pattern on sequencing read quality. The numbers of bases whose Phred scores exceed 30 (the Phred Q30 counts) within the first 1000 bases read are shown. Each of the four LNA incorporation patterns was applied to each of the six AT-rich sequencing primers as indicated below the graph; the data are from Table 3. The order of presentation within each primer group, from left to right, is Unmodified DNA (light gray); LNA-3′ (dark gray); LNA-5′ (white); LNA-Even (black).

primers with LNA at the 3′ position have been used for SNP detection by PCR (32). LNA-5′ nearly eliminated the mispriming that made the sequence unreadable for the other primers. Table 3 shows that the improvement is reflected in an average Phred Q30 count 60% higher than the average unmodified primer, as well as a decreased variance in the Q30 count. The effect of LNA-5′ was more pronounced for the ATM primers (for which the Q30 count nearly quadrupled) than for the MCP primers, because the unmodified MCP primers already had high Q30 counts, but in no case did LNA-5′ decrease performance. The results (Tables 1 and 3) from earlier sequencing reactions on the MCP1 primer confirm the superiority of the LNA-5′ pattern. In contrast, LNA-Even and LNA-3′ did not improve performance, yielding substantial mispriming and average Phred Q30 counts that were slightly less than those of unmodified primers. The same order of quality—LNA 5′ > Unmodified DNA > LNA 3′ > LNA-Even—was also seen in the Phred Q20 counts (data not shown). The LNA-5′ effect was presumably due to an increase in hybridization strength at the 5′ end of the primers that stabilized on-target hybridization and destabilized off-target 3′ mispriming.

ANOVA shows that the LNA-5′ effect is statistically significant

The ANOVA results (Table 4A) show that primer sequence [$F_{(5,48)} = 166.9$, $P < 0.0001$, where $F_{(5,48)}$ is the F-test statistic for 5 d.f. and 48 observations], LNA pattern [$F_{(3,15)} = 8.2$, $P = 0.0018$] and interaction between primer sequence and LNA pattern [$F_{(15,48)} = 14.0$, $P < 0.0001$] all have a highly significant effect on the Phred Q30 count. A significant effect of primer sequence is not unexpected. Primers of similar base composition but different base order often generate disparate Q30 counts or PCR product. This can be due to secondary structure, off-target hybridizations and the ability of the primer to be an efficient substrate for the polymerase. The significant interaction effect means that the effect of LNA incorporation on Q30 count depends on the primer sequence to which the pattern is applied.

Since the global test also identified a significant effect of LNA pattern on Q30 count, pair-wise comparisons were performed in order to determine which LNA patterns were significantly different from the unmodified DNA. The paired comparisons confirmed that the LNA-5′ primers generated significantly longer Q30 counts than the unmodified primers [$F_{(1,5)} = 6.58$, $P = 0.050$; data not shown]. However, the differences between the LNA-3′ primers and the unmodified primers [$F_{(1,5)} = 1.47$, $P = 0.280$] and the LNA-Even primers and the unmodified primers [$F_{(1,5)} = 3.76$, $P = 0.110$] were not significant (data not shown). Statistically, this lack of significance could be due to the small sample size and the large amount of interaction.

Selection and characterization of poorly-performing unmodified PCR primers

To generate test DNA primers that yielded poor PCR results, we started with the six AT-rich primers described above and extended them to the 5′ side to give primers with predicted melting temperatures of ~60.0°C, denoted with an '*L*' at the end of the primer name. Due to the AT-rich nature of

Table 4. ANOVA of LNA effects on DNA sequencing and PCR

Source of variation	Two-way ANOVA with replication (mixed model) SS[a]	d.f.[b]	MS[c]	F[d]	P-value	F crit[e]
(A) LNA incorporation significantly affects Phred Q30 count						
Primer sequence	4 102 578	5	820 516	166.9	<0.0001	2.41
Chemistry	1 692 039	3	564 013	8.2	0.0018	2.80
Interaction	1 030 538	15	68 703	14.0	<0.0001	1.88
Within	235 943	48	4915			
Total	7 061 098	71				
(B) Sequence and LNA incorporation interact to affect real-time PCR						
Primer sequence	158.3	2	79.15	45.38	<0.0001	3.32
Chemistry	335.9	4	83.97	4.31	0.0380	3.84
Interaction	155.8	8	19.47	11.16	<0.0001	2.27
Within	52.3	30	1.74			
Total	702.3	44				

(A) Primer sequence, LNA chemistry and an interaction between the two all significantly affect the Q30 count, as indicated by the P-value column. The underlying data is that of Table 3.
(B) The underlying data is that of Table 5.
[a]SS = Sum of squared deviations from the mean.
[b]d.f. = Degrees of freedom.
[c]MS = Mean square, SS/d.f.
[d]F = F-test statistic.
[e]F crit = the F-distribution critical value for achieving significance at $P = 0.05$.

Table 5. Real-time PCR is enhanced by the LNA-5′ incorporation pattern

Primer set[a]	Sequence (5′–3′)[b]	Length	T_M (°C) predicted reaction[c]	T_M (°C) predicted melt	T_M (°C) measured melt	ΔT_M (°C) melt	C_T[d]
MCP1L[a]							
DNA(n + 1)	GCAGCTTAAATTTTCTTGAAT	21	52.0	65.6	66.8	−1.2	30.1 ± 0.5
DNA(n)	CAGCTTAAATTTTCTTGAAT	20	48.4	62.1	63.8	−1.7	(34.8 ± 0.5)
LNA-3′	CAGCTTAAATTTTCT+TGA+AT	20	51.3	65.7	67.0	−1.3	(35.5 ± 0.4)
LNA-5′	+CA+GCTTAAATTTTCTTGAAT	20	51.1	65.0	65.4	−0.4	29.9 ± 0.4
LNA-Even	CA+GCTTAAATTTT+CTTGAAT	20	52.7	66.8	68.1	−1.3	29.4 ± 0.3
Reverse	CTAAGAAGCAGATAATGGTGTT	22					(36.3 ± 0.6)
ATM1L[a]							
DNA(n + 1)	GCTGAAGAGGAAGCATA	17	51.3	65.8	65.4	0.3	25.6 ± 0.2
DNA(n)	CTGAAGAGGAAGCATA	16	46.8	61.4	62.2	−0.8	(35.3 ± 0.7)
LNA-3′	CTGAAGAGGAA+GCA+TA	16	51.8	66.9	66.2	0.7	(>36)
LNA-5′	C+TGAA+GAGGAAGCATA	16	51.6	66.5	65.2	1.3	30.7 ± 1.2
LNA-Even	CTGAA+GAGGAA+GCATA	16	51.5	66.7	67.8	−1.1	(38.0 ± 1.0)
Reverse	AATGTCTTCCAAACAAATGTAAT	23					(>38)
ATM2L[a]							
DNA(n + 1)	GAAGCATATAAGTCAGAAT	19	47.7	61.6	62.9	−1.3	33.2 ± 0.9
DNA(n)	AAGCATATAAGTCAGAAT	18	45.6	60.0	61.4	−1.4	(36.7 ± 1.4)
LNA-3′	AAGCATATAA+GT+CA+GAAT	18	57.1	66.9	69.8	−2.9	(>38)
LNA-5′	AA+GCA+TATAAGTCAGAAT	18	50.1	64.9	60.0	4.9	(34.5 ± 1.4)
LNA-Even	AAGCA+TATAAGTCA+GAAT	18	50.0	65.2	66.7	−1.5	(>39)
Reverse	AATGTCTTCCAAACAAATGTAAT	23					(>38)

[a]The primers are extended versions of those in Table 1.
[b]LNA positions are underlined and preceded by + signs.
[c]PCR conditions are as described in Materials and Methods.
[d]The tabulated values are the cycle threshold (C_T) averages and standard deviations from three replicate real-time PCR experiments (Figure 3). C_T values in parentheses indicate values that are unreliable and were not used in quantitative analysis.

the target sequence, we were able to identify reverse primers for only three of the forward primers, MCP1L, ATM1L and ATM2L. We then performed real-time PCR with SYBR Green detection using sets of primers differing by 1 bp, as for DNA sequencing. These experiments identified the threshold forward primer that provided good PCR efficiency [denoted DNA(n + 1) in Table 5] and the next-shorter forward primer, DNA(n), which provided poor PCR efficiency.

We then incorporated LNA into the three unmodified DNA(n) primers using the LNA-5′, LNA-3′ and LNA-Even patterns as described above. The goal was to elevate each melting temperature to that of the DNA(n + 1) primer. This required three LNAs for the ATM2 LNA-3′ primer and two LNAs for all the others. We then performed real-time PCR experiments in triplicate with each of the five primers. All replicates were performed in the same plate. PCR experiments with just the master mix, containing the reverse primer, generated a baseline signal. As before, statistical analysis was by two-way ANOVA with replication.

The LNA-5′ incorporation pattern improves real-time PCR

Representative real-time PCR results are shown in Figure 3 and Table 5. The amplification plots of Figure 3 show

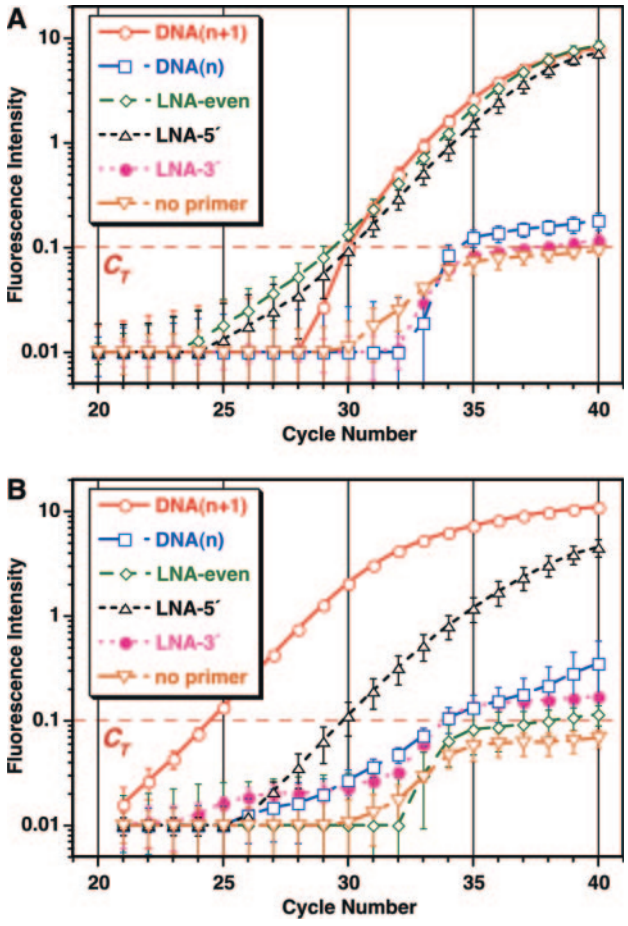

Figure 3. Real-time PCR using LNA-modified versus DNA primers. SYBR green fluorescence intensity (arbitrary units) is shown as a function of cycle number. (**A**) Shows the MCP1L forward primers and (**B**) shows ATM1L. The templates are PCR amplicons of the corresponding genes. The data points are the averages and standard deviations of triplicate reactions. For presentation on a log scale, values <0.01 (all were near background, ⩾−0.06) are displayed at 0.01, but with their actual standard deviations. The forward primers are as follows: open circles, DNA (n + 1); squares, DNA(n); filled circles, LNA-3′; upward-pointing triangles, LNA-5′; diamonds, LNA-Even; downward-pointing triangles, no forward primer.

SYBR Green fluorescence as a function of PCR cycle. Table 5 shows the corresponding cycle threshold (C_T); considering PCR amplification efficiency instead gave qualitatively consistent conclusions, but the C_T results are discussed here because they captured the qualitative observation that many of the amplification reactions never reached signal levels comparable to the on-target amplification. Figure 3A shows that for the MCP1L primers, the LNA-5′ and LNA-Even primers worked about as well as the efficient DNA(n + 1) primer. All three primers plateau at about the same level and cycle. In contrast, the LNA-3′ primer and the poorly-performing DNA(n) primer exhibited much less amplification. The C_T values for these primers were actually higher than in the master mix control, which contained only the reverse primer. C_T values of this magnitude are likely to be spurious and very low levels of amplification were observed.

Figure 3B shows that the DNA(n + 1) ATM1L primer outperformed all of its related LNA primers. The signal strengths of all but the ATM1L DNA(n + 1) primer are relatively low, as evidenced by C_T values of >30, compared to C_T values of 20–25 for a typical efficient PCR. The low signal strengths could be due to low primer melting temperatures of the forward primers (∼46.0–52.5°C) compared to the reaction temperature (60.0°C). Also, the melting temperature of the forward primers is much lower than the melting temperature of the reverse primers (∼60.0°C). This disparity may contribute to inefficiency. The 5′-LNA primer was, however, clearly superior to LNA-3′, LNA-Even and the DNA(n) primer. These latter reactions had high C_T values, comparable to the MCP1L LNA-3′, LNA-Even and master mix. The ATM2L primers displayed a pattern similar to that of the ATM1L primers (Table 5).

The general picture that emerges from these data is that incorporating LNA at or near the 5′ end of a PCR primer improves short PCR primers. The LNA-5′ pattern improved all of the DNA(n) primers, although not always to the same level as the DNA(n + 1) primers. It might be possible to improve the performance of the ATM1L and ATM2L LNA-5′ primers to the level of the DNA(n + 1) primer by adding additional LNAs and/or by using a different pattern of LNA-5′ incorporation. The real-time PCR results on the ATM primers demonstrate that raising primer T_M is not sufficient to ensure improved priming performance. We suspect that the ATM2L LNA-5′ primer may exhibit self-hybridization that hampers priming: given that LNA-G: DNA-T can form a stable mismatch (33), the +GCA+ TATAAGT portion could form a 10 bp helix with two internal A–A mismatches. It is also possible that PCR amplification is inhibited through LNA's effects on the polymerase, but as for sequencing, it appears that the LNA primers are capable of being extended even when they do not give amplifiable product.

The effect of the LNA-5′ pattern on PCR primers is significant

ANOVA was applied to the factors affecting cycle threshold (Table 4B). Since the high-C_T primers generated little or no specific product, the C_T values for these primers are unreliable. For primers where at least one replicate failed to attain the intensity threshold after 40 cycles, we do not report the standard deviation in Table 5. For ANOVA, we replaced these C_T values with a value of 40.

As observed for the sequencing primers, PCR primer sequence [$F_{(2,30)} = 45.38$, $P < 0.0001$], LNA pattern [$F_{(4,8)} = 4.31$, $P = 0.038$] and interaction between primer sequence and LNA pattern [$F_{(8,30)} = 11.16$, $P < 0.0001$] each have a significant effect on the PCR amplification. A significant effect of primer sequence on PCR is not unexpected, for the same reasons discussed above. The amplification plots showed that it was necessary to perform pairwise comparisons only for the LNA-5′ pattern, which revealed that the LNA-5′ primers generated significantly lower C_T values than DNA(n) [$F_{(1,2)} = 21.35$, $P = 0.044$].

Interaction between primer sequence and LNA pattern was highly significant in the global analysis and almost significant [$F_{(2,12)} = 3.00$, $P = 0.088$] in the pair-wise comparison between LNA-5′ and the unmodified primer described above. Although the interaction was not quite significant in the pair-wise comparison, we conservatively computed the

LNA-5′ versus unmodified pair-wise *F*-statistic by dividing the LNA pattern mean square by the interaction mean square, as recommended by Sokal and Rohlf (34) and described in Material and Methods.

DISCUSSION

Effect of LNA position

The sequencing and quantitative PCR results show that LNA-5′ is the best incorporation pattern for both methods. LNA-5′ primers were never worse than their unmodified counterparts. They nearly quadrupled the sequencing read lengths, as measured by Phred Q30 counts, of three primers designed for the ATM gene and they also generated more consistent Q30 counts. The LNA-5′ pattern also increased PCR yield relative to unmodified DNA of the same length. In contrast, the LNA-3′ and LNA-even incorporation patterns generated lower Q30 counts and higher C_T values than the unmodified primers, due in part to multiple sequencing ladders and non-specific PCR products. The LNA-Even appears to be worse than the LNA-3′ pattern, but the differences were not statistically significant.

These findings are sensible in view of the thermodynamics of primer hybridization and the mechanism of sequencing and PCR. In general, a primer that binds more strongly at its 5′ end should have better specificity than a primer that binds more strongly at its 3′ end. Partial off-target hybridization at the primer's 5′ end is not expected to generate product, but partial hybridization at the 3′ end may result in polymerase extension and thus non-specific product. Experimentally, Rychlik (35) found that high efficiency primers often have both 5′ hybridization that is stronger than 3′ hybridization and also moderate-strength 3′ hybridization. The 5′ partial hybridizations out-compete the 3′ partial hybridizations, while the moderate 3′ hybridization keeps the 3′ end of the primer hybridized to its target. When the GC% of the 3′ end becomes too high, the primers are susceptible to off-target priming.

Since LNA-5′ primers presumably hybridize more strongly at their 5′ ends, on-target hybridization is stabilized but off-target hybridization does not lead to product. In contrast, the LNA-3′ and LNA-Even primers stabilize off-target hybridization leading to non-specific product. They may also reduce on-target yield by diverting polymerase away from the target site. It is unlikely that the poor performance of the LNA-3′ and LNA-Even primers was due to inhibition of polymerase extension (36). In this case, clean but low-amplitude sequencing traces would be expected (low signal). Instead, we observed strong and jumbled traces (high noise), indicating false priming rather than inhibition.

Our finding that LNA-Even is the worst pattern is in contrast with Latorra's (9) conclusion that LNA-Even is best. We did observe that LNA-Even primers performed well some of the time and this may have been the case for the single primer that they studied. Finally, our finding that LNA-3′ primers performed less-well than conventional primers is consistent with Latorra's results (9).

The evidence suggests that LNA should never be placed near the 3′ end of sequencing primers or PCR primers unless it is necessary for methods like allele-specific PCR, for which LNA at the variable position has been reported to enhance allelic discrimination (10). This improved discrimination derives in part from LNA's destabilization of mismatches and in part from a decreased efficiency of the polymerase at an LNA end, as evidenced by an overall weaker signal as compared to perfect-match primers.

ANOVA research design

The decision to use ANOVA in the early stages of experimental design forced us to plan data analysis in advance, whereas otherwise it would not have been obvious how to fully populate the research design. The use of ANOVA here helped make it clear that the proper comparison of LNA primers is to unmodified primers of the same sequence, which was not done in several previous studies (9,14,15,37).

ANOVA has much to recommend it over the more commonly used paired *t*-test. While the two methods are mathematically equivalent for pair-wise comparisons, the paired *t*-test cannot provide a global analysis when there are more than two experimental conditions. The recommended method for ANOVA is to perform pair-wise comparisons only after the global analysis indicates significance. The paired *t*-test is not informative as to interactions among experimental dimensions and it does not provide the structure to objectively determine when to adjust *P*-values for multiple comparisons. ANOVA also provides guidance on the needed sample size and power given initial expectations for the magnitude of an effect.

In this study, we used Phred quality scores and cycle threshold (C_T) as quantitative measures of primer quality (38). These measures may be more accurate and precise than previous measures, such as read length or gel band density, and they are likely to become more widely-used in developing primer design rules. The use of triplicate data on a number of primer sequences is an expensive research design, but the resulting decrease in the variance added to the statistical power of the ANOVA. This depth of data coverage was made possible by defining a limited number of LNA patterns. The ANOVA allowed us to formally test for interactions between primer sequences and LNA incorporation pattern and distinguish it from the main effect of LNA incorporation pattern. The interaction was statistically significant and was not qualitatively obvious, and this work solidifies previous anecdotal evidence for such interactions (9,14,15,37).

Additional thermodynamic considerations for the design of LNA primers

The ideal thermodynamic dataset for use in primer design algorithms would be a complete description of all possible LNA–DNA mixmers hybridized to their complements and to all possible mismatches. Such a dataset is not available, but existing data allows us to propose some general rules for LNA-enhanced primer design.

Our database of thermodynamic parameters for single, internal LNA incorporation (22) generated melting temperature predictions that were accurate enough to be useful for primer design, but not as accurate as the predictions for unmodified primers. In general, LNA pyrimidines provide more stabilization than purines, with LNA-A being the least stabilizing base. The most inaccurate predictions tended to

occur in primers that have LNA in terminal or penultimate positions. These results suggest that the generation of thermodynamic parameters for LNA incorporation at terminal and penultimate positions will enable accurate T_M predictions for a larger variety of primers. Preliminary results (M. F. Samala, J. Levin, R. J. Peterson and J. D. Kahn, unpublished data) suggest that 5′ terminal LNA provides only a slight increase in stability and the 5′ penultimate position less stabilization than internal positions. The accuracy of T_M predictions should also increase with the availability of thermodynamic data for multiple LNA incorporations and for mismatches. While mismatch data are obviously relevant to SNP methods, they are also useful for predicting LNA stabilization of off-target hybridization. In this regard, LNA-G:DNA-T mismatches appear to be especially problematic (33).

Accurate prediction of melting temperature for primers that contain any modified chemistry at any position will require generation of a comprehensive database of thermodynamic parameters: single and multiple internal incorporations; 5′ and 3′ terminal and penultimate incorporations; and mismatches. Thermodynamic data will improve design for any method that uses hybridization of modified nucleic acid chemistries.

Applications of LNA primers

The design of LNA primers can be complicated because of the stabilization of both on- and off-target hybridization. Optimal LNA primer design will require assay design software that applies positional and thermodynamic rules. Moreover, it is important to establish the conditions for which LNA is beneficial, since it is substantially more expensive than traditional DNA and RNA chemistries. Here we consider only the thermodynamic aspects of LNA use; of course, its ease of synthesis, chemical stability and resistance to nucleases are also critical in many applications.

LNA is likely to improve performance most significantly when primer hybridization would otherwise be inefficient. This occurs most frequently in AT-rich regions, where unmodified primers of limited lengths have melting temperatures that are too low to hybridize well under standard reaction temperatures. LNA incorporation should then improve sequencing quality and PCR yield and we have demonstrated that poorly-performing primers can be rescued most effectively using the LNA-5′ incorporation pattern. LNA might also be useful for sequencing GC-rich templates on which the available priming site is short, as it might be able to increase primer T_M to allow the use of higher primer annealing and extension temperatures without increasing primer length.

LNA-modified short primers can be positioned with more versatility than DNA primers. Exact placement is essential to methods that target a specific feature with stringent primer-placement rules, such as MassArray SNP genotyping (1) and pyrosequencing of potentially methylated CpG sites (39). Short primers are better able to avoid tandem and interspersed repeats, as well as sequences that generate primer hairpins or primer-dimers. These regions have been problematic for the new sequencing technologies.

Summary

LNA incorporation near the 5′ end of sequencing and PCR primers improves performance, but LNA near the 3′ end and LNA evenly spaced throughout the primers do not. We suggest that LNA-5′ increases primer stability at its target site without enhancing extension of primers at off-target sites, whereas LNA-3′ and LNA-even promote mispriming. ANOVA methodology enabled us to rigorously quantify the effects of primer sequence, LNA position and the interaction between the two. The positional rules derived here, as well as thermodynamic parameters for any kind of LNA incorporation, are essential for software that designs LNA primers for sequencing and PCR.

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Conflict of interest statement. Celadon Laboratories sells primer design software and services that consider LNA-modified oligonucleotides.

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