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Design considerations and effects of LNA in PCR primers

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Abstract

The effects of comprehensive LNA substitution in PCR primers for amplification of human genomic DNA targets are presented in this report. Previous research with LNA in other applications has shown interesting properties for molecular hybridization including enhanced specificity in allele-specific PCR. Here we systematically modified PCR primers and conditions for the human genomic DNA targets APOB and PAH, along with a β-globin amplification control, to study whether the number and position of LNA residues improves or diminishes amplification sensitivity and specificity. It was observed that the design rules for LNA substitution in PCR primers are complex and depend upon number, position and sequence context.

Technical advantages were seen when compared to DNA controls for the best LNA primer designs, which were typically one to a few centrally located LNA residues. LNA advantages include increased maximum annealing temperature (T_{max}) and increased signal with limiting primer or Taq DNA polymerase. Several well-characterized designs exhibited different efficiencies with different brands of hot-start enzymes. Many shorter LNA primers were found to be functional compared to same-length non-functional native DNA controls. These results show that LNA-substituted PCR primers have potential for use in difficult PCR techniques, such as multiplex amplification at higher T_{max}, once firm LNA primer design rules are established.

Keywords: Locked nucleic acid (LNA); Polymerase chain reaction (PCR); Apolipoprotein B (APOB); Hybridization; LNA primer design

1. Introduction

The enormous utilization of the polymerase chain reaction (PCR) in molecular research and diagnostics has been driven by scientific ingenuity and the remarkable sensitivity and specificity of the technology [1]. Incremental PCR improvements and applications are myriad and continue to evolve. Likewise, a novel synthetic nucleic acid chemistry called Locked Nucleic Acid (LNA) has been described [2–5] and shown to facilitate performance in several types of applications. Known attributes of LNA oligonucleotides include their ability to bind complementary nucleic acids with higher affinity and specificity, and the ability to synthesize virtually any primer or probe sequence desired in combination with DNA amidites. These strengths have been demonstrated in hybridization-based assays for polymorphism detection by hybrid probe capture in micro well plates [6,7], fluorescence polarization [8], decoy oligonucleotides [9], antisense applications [10,11] and the like.

Our previous research showed clearly that 3′ LNA residues improved the specificity of allele-specific PCR primers compared to native DNA primers [12,13]. This LNA enhancement of specificity was observed to occur over a wide range of PCR conditions and for multiplex reactions, presumably due to the altered interaction of Taq DNA polymerase with the more rigid structure of the LNA residues. Support for this hypothesis is gleaned from duplex formation kinetic studies, which showed that a slower dissociation rate for LNA-containing complexes was responsible for differences in hybridization performance from native DNA [14].

A simple and logical extension of the work with both PCR and LNA primers was to test them in concert in a thorough and systematic fashion. In this report we investigated the effects of LNA substitution throughout PCR primers for two well-characterized human polymorphic targets (along with a β-globin amplification control) in an attempt to elucidate design rules. These

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variable number tandem repeat targets are 3′ hyper variable regions near two human genes, apolipoprotein B (APOB) and phenylalanine hydroxylase (PAH). From any given human DNA sample, these targets will generate two amplicons from among many possible alleles containing 15–30 bp repeats and were previously optimized and validated for human identification analysis [15].

These PCR targets were selected for their sensitivity to variations in reaction and thermal cycling conditions. The DNA primers have melting temperatures (T_m) in the mid-50s (°C) to allow any effects of LNA-substitution to be observed. Here, we extensively varied the LNA content throughout both primers of these two pairs and tested them under various PCR conditions to measure their performance vs. DNA controls and determine whether specific design rules could be deduced. LNA primer designs included various numbers and positions of LNA residues, along with variations in base specificity and primer length. Virtually every base substitution position was analyzed for APOB primers with two to seven LNA residues. Primer pairs in all possible combinations were tested, including leaving one primer of each pair as DNA while modifying the second and with both primers modified with LNA. PCR variables analyzed that could be technically advantageous included: improved yield with lower amounts of Taq DNA polymerase; lower required primer or template amounts; wider range of working annealing temperatures; shorter primers with LNA compared to DNA to find minimum lengths required, etc. This work has shown several interesting and advantageous properties of LNA-containing PCR primers, along with general principles for preliminary LNA primer design rules.

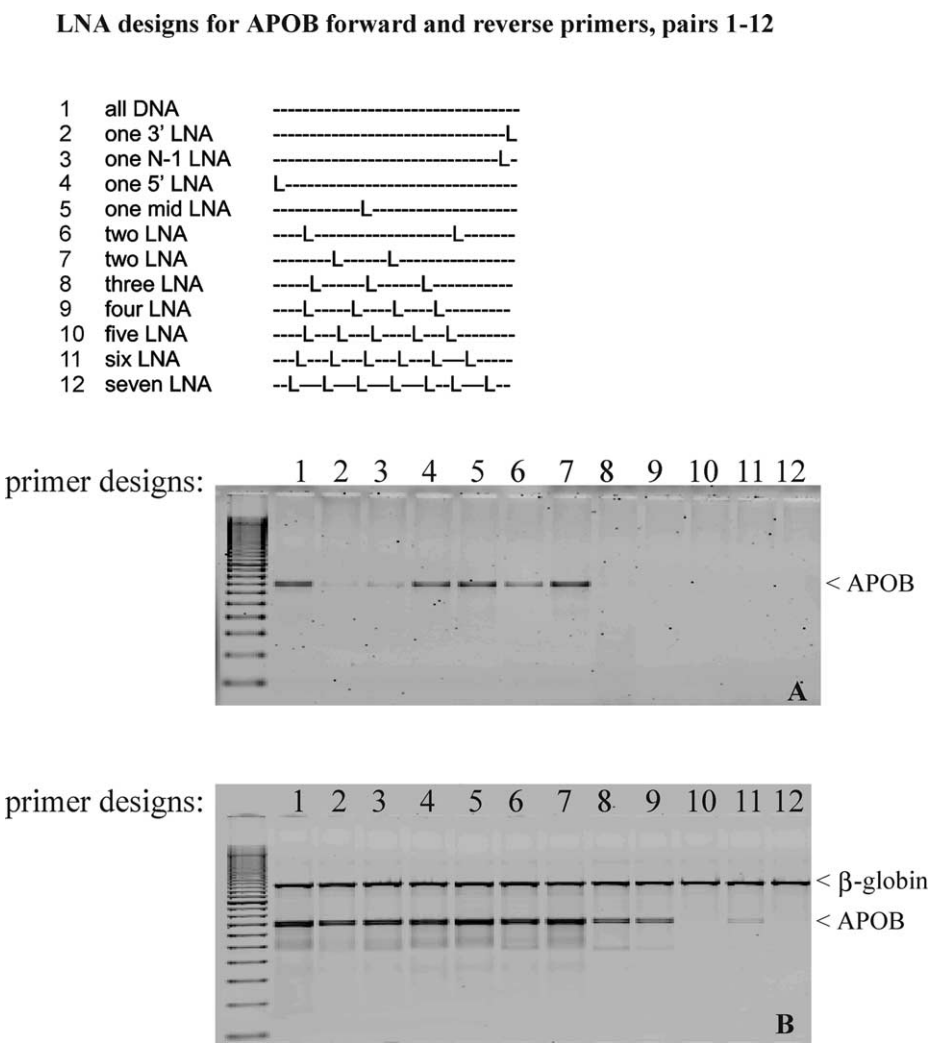


Fig. 1. Effects of LNA substitution in APOB forward and reverse primers. The top schematic shows the general design principles for both forward and reverse primers for APOB pairs 1–12. The actual sequences are listed in Table 1. Panel A shows the amplicon results from these pairs using AmpliTaq Gold DNA polymerase, while panel B shows results with FastStart Taq (along with a β-globin amplification control band).

2. Materials and methods

All oligonucleotides used in this study were synthesized by Proligo LLC and desalted prior to use. PCR primer sequences for APOB primers with specific LNA substitutions are listed in the Figures, while the sequences for unmodified PAH and β-globin primers are as follows: P1-5′GCTTGAAACTTGAAAGTTGC; P2-5′GGAAACTTAAGAATCCCATC; β1-TGGTAGCTGGATTGTAGCTG; β2-GGTTGGCCAATCTACTCCCAGG. PCR amplification was done using a block thermal cycler (DNA Engine™, MJ Research) programmed for 30 cycles of 94 °C for 35 s, 59 °C for 35 s and 72 °C for 30 s following a 5 min Taq activation step. Standard reactions with APOB primers contained 1X reaction buffer, 200 μM dNTPs, 2.5 mM $MgCl_2$, 20 ng human genomic DNA, 1 unit of Taq DNA polymerase, 0.5 μM primers and 0.05 μM β-globin control primers. Titration experiments of PCR reactants with LNA and DNA primers were mainly done using AmpliTaq Gold® DNA polymerase (Applied BioSystems), unless otherwise specified with FastStart™ Taq polymerase (Roche). The following ranges were used in the PCR titration experiments: annealing temperatures—50, 50.5, 51.5, 53.2, 55.5, 58.4, 61.8, 64.6, 66.8, 68.4, 69.6 and 70.0 °C; enzyme amount—0.1, 0.25, 0.5, 1.0 and 1.5 units; primer concentrations—0.1, 0.25, 0.5, 0.75 and 1.0 μM; $MgCl_2$ concentrations—1, 2, 3, 4, 5, 6, 7 and 8 mM and template amounts—0.2, 2.0, 20, and 200 ng. Gel electrophoresis was done using 2% agarose gels with standard ethidium bromide staining. Image scanning and densitometry was done with a Typhoon 8600 scanner and ImageQuant software (Amersham Biosciences).

3. Results

Initially eleven different APOB primer pairs were designed (plus one DNA control) with various LNA

APOB LNA-modified primer pairs in Figure 2			
			APOB
Primer pair	forward sequence	reverse sequence	Tmax
A	gttcctcaggatcaaagtatgtac	ggagaaattatggagggaaat	61.8
B	gttcctcaggatcaaagtatgta**C**	ggagaaattatggagggaaa**T**	61.8
C	gttcctca**G**gatcaa**A**gtatgtac	ggagaa**A**ttatgg**A**gggaaat	66.8
D	gttc**C**tcag**G**atca**A**agta**T**gtac	gga**G**aaat**T**atg**G**agg**G**aaat	66.8
E	gttcctcag**G**atcaaagtatgtac	ggaga**A**attatggagggaaat	64.6
F	gttcctc**A**ggatcaaa**G**tatgtac	ggagaaa**T**tatgga**G**ggaaat	64.6
G	gttcctcagga**TC**aaagtatgtac	ggagaaatta**TG**gagggaaat	66.8
H	gtt**C**ctcagga**T**caaagta**T**gtac	gga**G**aaatt**A**tggagg**G**aaat	64.6

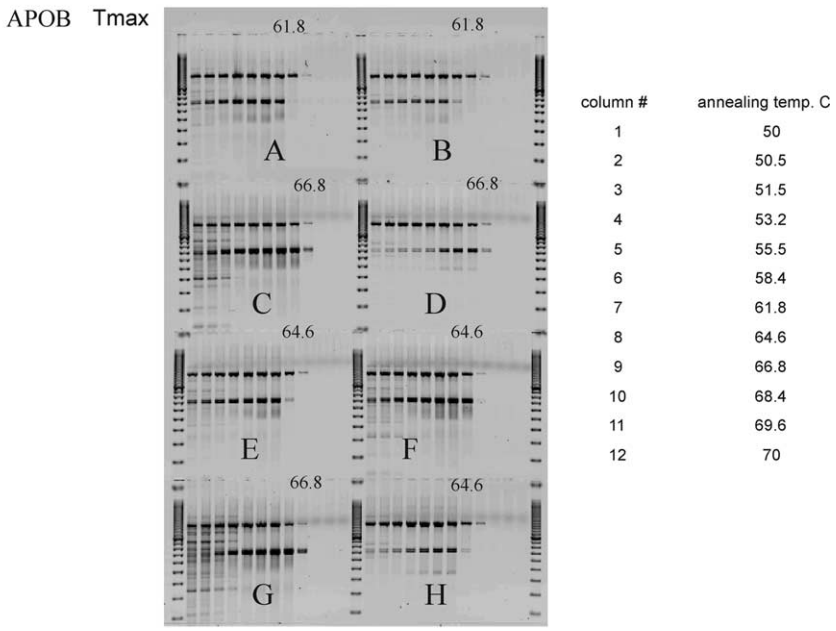


Fig. 2. APOB + β-globin annealing gradient with eight primer pair designs. Forward and reverse primer sequences are listed for pairs A–H with LNA residues in bold capital letters. The agarose gel image below shows twelve conditions for each primer pair A–H. These conditions were different in the annealing temperature of each well of the PCR plate as shown to the right of the image (ranging from 50 to 70 °C). The upper bands in each set are the β-globin amplification controls (1327 bp) while the lower doublet bands are the APOB amplicons (~700 bp). The number above each set is the highest annealing temperature that produced a visual APOB band, and is defined as the T_{max} for that PCR.

substitution patterns and extensively characterized in this study (see Fig. 1 schematic). These APOB primer designs looked at one LNA residue in key positions (3′, 3′[$n-1$], 5′, central) along with between two to seven LNA modifications for both forward and reverse primers. Testing of multiple genomic DNA samples confirmed that several LNA designs performed as well or better than native DNA primers, while others failed outright. Fig. 1 clearly shows that the 3′ LNA primer pair produces a relatively weaker signal (lanes 2), while other pairs with one or two modifications gave generally strong amplicons for the bi-allelic APOB target of ~700 bp (lanes 5 and 7). These two alleles (not completely resolved in Figs. 1 and 2) in the template standard have 34 and 36 known 15 bp repeats and thus their amplicons differ in size by only 30 bp. Primers with more than two LNA residues failed to yield APOB product with AmpliTaq Gold, but did so with 3, 4 and 6 (weakly) residues using FastStart Taq in Fig. 1. The 1.3 kb β-globin amplification control was used in panel B, while results without this control (such as panel A) were confirmed with numerous templates and repetitions.

Pixel density analysis of APOB amplicons from the DNA control and LNA primer designs 1–12 are presented in Table 1 along with the specific sequences examined. Comparison of the band intensities of the control DNA primer pair (#1) to the LNA pairs shows that designs 5 and 7 generated stronger amplicons than for the DNA control with both versions of hot-start Taq. Although FastStart Taq DNA polymerase is more permissive to higher levels of LNA substitution than AmpliTaq Gold, the pattern of pixel data in Table 1 is very similar for both enzymes. Other PCR optimization experiments done with AmpliTaq Gold are summarized in Table 1 and show that the best LNA primer designs (5 and 7) exhibited technical advantages compared with the DNA control pair: the ability to successfully use lower amounts of Taq DNA polymerase (0.1 vs. 0.25 units) or concentration of primer (0.1 vs. 0.25 μM). A FastStart Taq enzyme titration experiment with APOB primer designs 1–9 showed that with 0.5 units, several LNA primer pairs (especially #7) yielded product while the DNA control pair did not (data not shown).

Other parameters such as optimal $MgCl_2$ levels (2.5 mM) and minimum human genomic DNA amount (200 pg) were equivalent between the DNA control and most LNA designs. Conversely, the worst LNA primer design (one 3′ residue) exhibited higher limiting levels of enzyme, primer, $MgCl_2$ and template than the DNA control primer pair. LNA design #8 (with three LNA residues) failed to produce APOB amplicons during standard or optimization conditions, therefore the rest of the designs with higher LNA content were not assessed by parameter titration using AmpliTaq Gold.

The LNA-containing primers operate under a wider range of effective annealing temperatures than comparable DNA primers. Fig. 2 is a representative annealing gradient result showing that LNA primers have approximately a 3–5 °C wider range of maximum effective annealing temperature (termed T_{max}) than DNA controls. We defined T_{max} as the highest annealing temperature that still yields a detectable amplified product. The LNA primer pairs tested in this example included pairs 2 and 7 (B and C, respectively) along with additional designs with 2–4 LNA residues, which all performed well under standard conditions. Fig. 2 shows eight panels (A–H) from the same PCR experiment with

Table 1
Characterization of designs 1–12 for LNA-containing APOB primer pairs. Primer sequences for APOB pairs 1–12 are listed with LNA residues in bold CAPS. Definitions of T_{max}, E_{min}, P_{min}, M_{min} amd D_{min} are given below the table for titration experiments done with AmpliTaq Gold enzyme. The last two columns give pixel data from triplicate densitometry measurements of APOB amplicons generated with both AmpliTaq Gold (ATG) and FastStart Taq (FST), respectively

Pair	Forward sequence	Reverse sequence	T_{max}	E_{min}	P_{min}	M_{min}	D_{min}	Pixels (ATG)	Pixels (FST)
1	gttcctcaggatcaaagtatgtac	ggagaaattatggagggaaat	61.8	0.25	0.25	1.5	0.2	987.5	30,164
2	gttcctcaggatcaaagtatgta**C**	ggagaaattatggagggaaa**T**	58.4	1.00	0.50	2.5	20.0	47.5	17,818
3	gttcctcaggatcaaagtatgt**A**c	ggagaaattatggagggaa**A**t	61.8	0.25	0.25	2.0	2.0	104.5	25,428
4	**G**ttcctcaggatcaaagtatgtac	**G**gagaaattatggagggaaat	64.6	0.10	0.25	1.5	0.2	737.2	27,772
5	gttcctcaggatca**A**agtatgtac	ggagaaatta**T**ggagggaaat	64.6	0.10	0.25	1.5	0.2	1023.6	33,142
6	gtt**C**ctcaggatcaaagtat**G**tac	gga**G**aaattatggaggg**A**aat	64.6	0.25	0.25	1.5	2.0	339.1	27,953
7	gttcctca**G**gatcaa**A**gtatgtac	ggagaa**A**ttatgg**A**gggaaat	66.8	0.10	0.10	1.5	0.2	1179.1	33,158
8	gttcct**C**aggat**C**aaagt**A**tgtac	ggag**A**aatta**T**ggag**G**aaat	61.8	1.00	x	x	x	19.4	14,313
9	gttc**C**tcag**G**atca**A**agta**T**gtac	gga**G**aaat**T**atg**G**agg**G**aaat	NT	NT	NT	NT	x	0	9,483
10	gtt**C**ctc**A**gga**T**caa**A**gta**T**gtac	gga**G**aa**A**tta**T**gga**G**gg**A**aat	NT	NT	NT	NT	x	0	172
11	gtt**C**ct**C**agg**A**tc**A**aag**T**at**G**tac	gg**A**ga**A**at**T**at**G**ga**G**gg**A**aat	NT	NT	NT	NT	x	0	1,516
12	gt**T**cc**T**ca**G**ga**T**ca**A**ag**T**at**G**tac	gg**A**ga**A**a**T**ta**T**g**G**ag**G**ga**A**at	NT	NT	NT	NT	x	0	215

T_{max} = maximum observed annealing gradient temperature (°C). E_{min} = lowest amount of enzyme (AmpliTaq Gold) yielding amplicons (units). P_{min} = lowest primer concentration yielding visible amplicons (uM). M_{min} = lowest level of $MgCl_2$ required for visible amplicons (mM). D_{min} = lowest template amount required for observed amplicons (ng). Pixels = denitonmetry values (in thousands) from three templates in triplicate (Fig. 1) for AmpliTaq Gold (ATG) and FastStart Taq (FST).

two variables, primer pair identity and annealing temperature.

The β-globin primers in Fig. 2 exhibited a constant T_{max} of 66.8 °C throughout reactions A–H (top band). The DNA control pair and 3′-LNA primers (B) give a T_{max} of ~62 °C. One or two LNA residues placed in the middle of each primer (E, F) increases T_{max} to ~64.5 °C, while prior design #7 (C), two LNA substitutions in a row (G) or four which are widely spaced (D) increased the T_{max} to ~67 °C. However, in one case, three spaced LNA residues (H) yielded a T_{max} of only ~64.5 °C, implying a sequence context dependence that extends beyond simply the number of residues substituted. This increased T_{max} with LNA primers was seen for the APOB products both with and without (data not shown) co-amplification of the β-globin primer pair. The T_{max} values produced should also be viewed in the context of the wide steps in the annealing gradient at the cut-off

Shorter APOB primer sequences in Figure 3

Pair ID	forward sequence	reverse sequence	Pair ID	forward sequence	reverse sequence
C	gttcctcaggatcaaagtatgtac	ggagaaattatggagggaaat			
L1	tcctcaggatcaaa**G**tatgtac	gagaaattatgg**A**gggaaat	L9	gttcctcaggatc**A**aagtatg	ggagaaattatg**G**aggga
D1	tcctcaggatcaaagtatgtac	gagaaattatggagggaaat	D9	gttcctcaggatcaaagtatg	ggagaaattatggaggga
L2	cctcaggatc**A**aagtatgtac	gagaaat**T**atggagggaaat	L10	gttcctcagga**T**caaagtatgt	ggagaaatt**A**tggaggg
D2	cctcaggatcaaagtatgtac	gagaaattatggagggaaat	D10	gttcctcaggatcaaagtatgt	ggagaaattatggaggg
L3	cctcag**G**atcaaagtatgtac	agaa**A**ttatggagggaaat	L11	gttcctca**G**gatcaaagtatgt	ggagaaa**T**tatggaggga
D3	cctcaggatcaaagtatgtac	agaaattatggagggaaat	D11	gttcctcaggatcaaagtatgt	ggagaaattatggaggga
L4	**T**cctcaggatcaaagtatgtac	a**G**aaattatggagggaaat	L12	gttcct**C**aggatcaaagtatg	gga**G**aaattatggaggg
D4	tcctcaggatcaaagtatgtac	agaaattatggagggaaat	D12	gttcctcaggatcaaagtatg	ggagaaattatggaggg
L5	cctca**G**gatcaa**A**gtatgtac	aa**A**ttatgga**G**ggaaat	L13	gttc**C**tcaggat**C**aaagtatg	gg**A**gaaatt**A**tggagg
D5	cctcaggatcaaagtatgtac	aaattatggagggaaat	D13	gttcctcaggatcaaagtatg	ggagaaattatggagg
L6	cagg**A**tc**A**aagtatgtac	gaaat**T**atg**G**agggaaat	L14	gttcc**T**cag**G**atcaaagta	gga**G**aaa**T**tatggagg
D6	caggatcaaagtatgtac	gaaattatggagggaaat	D14	gttcctcaggatcaaagta	ggagaaattatggagg
L7	tcagga**TC**aaagtatgtac	aaatta**TG**gagggaaat	L15	gttcctca**GG**atcaaag	ggaga**AA**ttatggagg
D7	tcaggatcaaagtatgtac	aaattatggagggaaat	D15	gttcctcaggatcaaag	ggagaaattatggagg
L8	ctcaggatc**AA**agtatgtac	gaaattat**GG**agggaaat	L16	gttcc**TC**aggatcaaag	gga**GA**aattatggaggg
D8	ctcaggatcaaagtatgtac	gaaattatggagggaaat	D16	gttcctcaggatcaaag	ggagaaattatggaggg

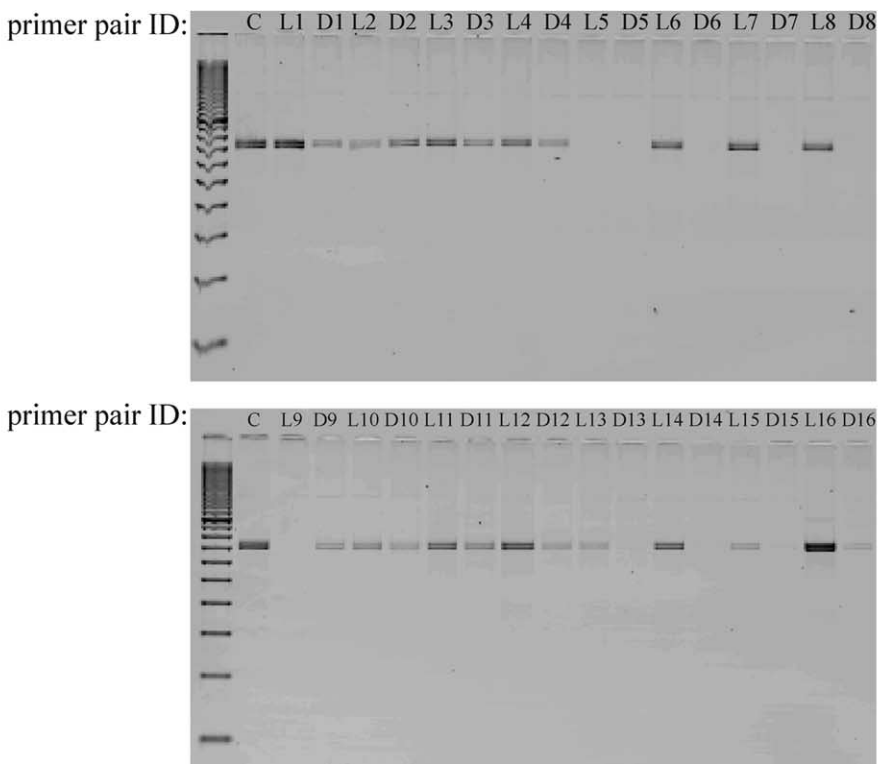
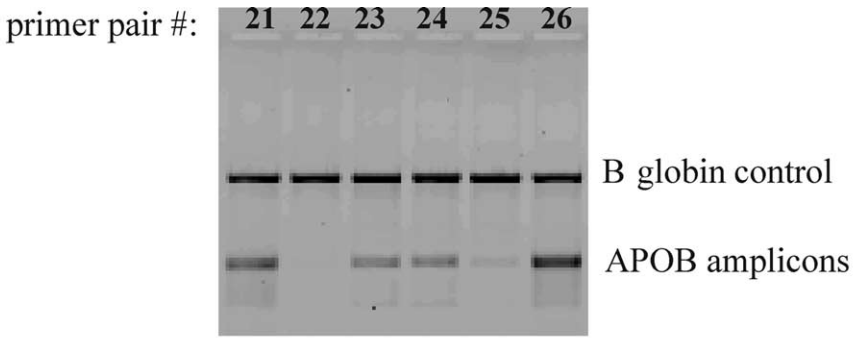


Fig. 3. Comparison of shorter APOB LNA and DNA primers. The table above shows 16 LNA and DNA short APOB forward and reverse primers with the length of each noted. LNA residues are indicated in bold CAPS. Pair C is the full-length DNA primers from Fig. 1 used as a control. The agarose gel image below shows the doublet APOB amplicons produced for each pair along with band sizing ladder indicating the correct products.



Primer pair #	Forward sequence	Reverse Sequence
21	gttcctcaggatcaaagtatgtac	ggagaaattatgga**G**ggaaat
22	gttcctcaggatcaaagtatgtac	ggagaaattatggag**G**gaaat
23	gttcctcaggatcaaagtatgtac	ggagaaattatggagg**G**aaat
24	gttcctcaggatcaaagtatgtac	ggag**A**aattatggagg**G**aaat
25	gttcctcaggatcaaagtatgtac	ggaga**A**attatggag**G**gaaat
26	gttcctcaggatcaaagtatgtac	ggagaaa**T**tatgga**G**ggaaat

Fig. 4. LNA design impact in six APOB primer pairs. The sequences of the forward and reverse primers are shown below the gel image with LNA residues indicated in bold CAPS. The gel image shows the APOB and β-globin amplification products from primer pairs 21–26 as indicated.

temperatures as shown in Fig. 2. It is also interesting to note the differences in non-specific amplification products observed between LNA primer pairs C and G in Fig. 2, although the designs are completely different.

A large number of additional LNA primer designs were analyzed for both APOB and PAH targets, including pairs with forward and reverse primer fixed as DNA to determine the LNA effects in each oligonucleotide. It was seen that the two primers were not equal in terms of their LNA substitution effect: one of each pair was dominant, i.e. modification of the APOB forward primer had less effect on performance than the reverse and visa versa for the PAH pair (data not shown). Fig. 3 shows a comparison of shorter DNA and LNA-containing APOB primers with the sequences listed. The primer length ranges from 17–24 nucleotides for the forward and 16–21 for the reverse primer, with one to two LNA residues per primer. Several shorter LNA-containing primers gave signal comparable to the full length DNA control, and 13 of 16 pairs tested gave stronger signal than same-length DNA controls. Among these pairs, six LNA-modified shorter primer pairs yielded APOB products where none was evident for the corresponding DNA pairs (Fig. 3, pairs 6, 7, 8, 13, 14 and 15).

One example of the positional effects observed in LNA primer design is shown in Fig. 4. Here the pairs are designed with the APOB forward primer fixed as DNA and the reverse primer modified at different positions with LNA. When looking at the sequence context of LNA substitution in a string of three G bases towards the 3′ end, it is evident that LNA position is critical for amplicon production. One LNA G-base at the 5th position from the 3′ end (middle of the three G bases) is deleterious to the PCR reaction (pairs 22 and 25), while substitution at either of the other two G bases is not. The best motif yielding the highest band intensity of this set is pair 26, which also has another upstream LNA substitution.

4. Discussion

This report details progress in understanding the performance and design rules of LNA-containing primers in PCR. Several distinct advantages of LNA primers compared with DNA controls were observed along with complexity in determining successful design specifications. LNA-containing PCR primers exhibited higher maximal annealing temperatures (T_{max}) and improved performance with shorter primers compared to same-length DNA controls. Certain LNA primers functioned better than comparable DNA primers at low levels of Taq DNA polymerase, which can potentially increase the cost-efficiency of PCR (especially as increased availability reduces the cost of LNA primers). We also observed a reduction in limiting LNA vs. DNA primer concentration required for pairs that were designed well.

The screening and titration results with the initial 11 LNA-modified APOB primer pairs suggested generally that too much LNA substitution in PCR primer sequences was detrimental and that positioning was an important

consideration. Multiple figures show optimal results with 1–3 LNA residues, some adjacent and others spaced apart, centrally located within the primer sequence. This confirms our prior work showing enhanced 3′ LNA primer specificity with no increase in amplicon yield in allele-specific PCR [12,13]. Fig. 4 shows clearly the complexity of sequence context in LNA primer design and how slight variations in LNA position yield different results. This is best accomplished experimentally until a more robust design algorithm is developed.

These results with superior LNA-containing primers (e.g. pair #7 in Fig. 1) suggest that either they bind better to their targets or are superior substrates for Taq DNA polymerase than comparable DNA control primers. Indeed, the LNA effects are likely the result of alteration of kinetics in the molecular interactions of primer with Taq DNA polymerase and/or target during the annealing step of the PCR process. An experiment to assess the ability of LNA primers to produce PCR products using fewer cycles of PCR than for DNA controls did not show any LNA advantage.

An extension of the T_{max} range was seen with many LNA primer pairs compared to DNA primers as shown in Fig. 2. This finding offers a strong advantage to facilitate the optimization of multiplex PCR applications, where several targets are tested under one PCR condition and higher specificity generally occurs at higher annealing temperature. This increase in T_{max}, combined with that from the improved performance of shorter LNA primers compared with DNA controls, hint at some broad rules governing the effect of LNA substitution in PCR primers and the relationship of T_m to T_{max}.

The primary goal of this report was to assess various designs to determine whether LNA incorporation in PCR primers offers any improvements over DNA primers, including if various PCR conditions could be relaxed or tolerated. The data presented here suggests that uncovering successful LNA primer design rules will be complex and that PCR performance may be affected by variables such as which specific hot-start Taq DNA polymerase enzyme is used. Given the previous advantages seen in the enhancement in specificity with allele-specific PCR and improvements in sensitivity shown here, further analysis of LNA primers is a worthy research goal to further the potential utility and efficiency of PCR.

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