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Method

Locked nucleic acids in PCR primers increase sensitivity and performance

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Abstract

The incorporation of locked nucleic acids (LNAs) into oligonucleotide primers has been shown to increase template binding strength and specificity for DNA amplification. Real-time PCR and DNA sequencing have been shown to be significantly enhanced by the use of LNAs. Theoretically, increasing primers' binding strength may also increase the sensitivity of conventional PCR, reducing minimum template requirements. We compared LNA–modified PCR primers with their standard DNA counterparts for amplification sensitivity with template amounts as low as 5 pg. Although the results are highly dependent on the design of the LNA primers, large increases in peak height can be achieved from as little as 75 pg, as well as clearer and more complete profiles. Increased amplification success with lower template amounts may also be seen. Additionally, the use of LNAs can enhance multiplexing. Thus, incorporating LNAs into PCR primers can increase amplification success, sensitivity, and performance under a wide range of conditions.

Keywords: Locked nucleic acids; Low-copy number; Multiplexing

Locked nucleic acids (LNAs) are a novel nucleic acid analog and are a promising tool for increasing oligonucleotide hybridization strength and specificity. The LNA bases can be incorporated into any DNA or RNA oligonucleotide and induce a conformational change in the local helix [1]. This altered state provides the LNA bases with stronger binding strength for complementary sequences [2,3], greater mismatch discrimination [4], and enhanced duplex formation [5]. These features increase amplification success when LNAs are incorporated into oligonucleotides and also increase duplex melting temperatures, which enable probes and primers to be shortened and give greater specificity [6]. Applications for LNAs to date include allele–specific PCR, TaqMan and Molecular Beacon probes, real-time PCR probes, antisense oligonucleotides, microarray probes, and PCR primers [see [7–9] for reviews].

The incorporation of LNA nucleotides into real-time PCR probes and primers decreases C_t values significantly [10,11], with a corresponding increase in amplification efficiency. For standard PCR, LNA modifications increase the specificity of the amplification, resulting in improved sequencing read quality [5], and can reduce the amount of template required by at least 10-fold [5,10]. For many applications, the ability to detect these lower amounts, even as little as one or two copies of a target sequence, would be highly desirable. Currently, optimizing standard PCR amplification protocols for low template levels (less than 100 pg) can take considerable effort and is impossible for some primer sets. However, the decrease in template requirements for real-time PCR using LNA oligonucleotides suggests that they may also be beneficial for standard PCR. Accordingly, we have incorporated LNA bases into currently used PCR primers. For our purposes (forensic DNA profiling), a decrease in template requirements would be highly valuable for trace DNA samples, i.e., those containing 100 pg or less of genomic DNA. However, any modification must maintain accurate and reproducible results and, ideally, for the forensic community, be capable of being incorporated within a multiplex system.

We compared LNA–containing primers (hereafter referred to as "LNA primers") with their standard DNA primer counterparts ("DNA primers") for amplification sensitivity and success at low template levels and examined the multiplexing ability of

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Table 1
Average optimal range for amplification parameters, as calculated using Taguchi orthogonal array design and level average analysis

Component	Locus	DNA primer sets	LNA primer sets
Primer concentration (μM)	FGA	0.08	0.2
	D13	0.1	0.1
	D7	0.1	0.15
	D18	0.2	0.4
MgCl concentration (mM)	FGA	0.8	0.4
	D13	0.4	0.7
	D7	0.5	0.4
	D18	0.4	1.0
Annealing temperature (°C)	FGA	2	8
	D13	3	8
	D7	3	4
	D18	2	3

each set, to determine if the LNA modifications are suitable for PCR primers used in DNA STR profiling.

Results

Optimization of PCR conditions

Each primer pair of the four STR loci chosen for study (FGA, D7S820, D13S317, and D18S51) had a different predicted melting temperature (T_m). Also, the addition of LNA bases to the primers increased the predicted T_m by between 1 and 5°C. Therefore, each primer pair was optimized separately, which enabled fair and accurate comparisons of the primers. A Taguchi factorial array design procedure was used [12,13], which minimized the number of reactions needed to only 16 reactions for each primer pair. From the two sequential Taguchi arrays (examining $MgCl_2$ and primer concentrations and annealing temperature), it was possible to find the most favorable conditions for each amplification. These experiments revealed that the LNA primers can have wider optimal ranges for each parameter than the DNA primers (Table 1).

Low-copy-number singleplex amplifications

To investigate if LNAs could decrease the minimum template requirements for successful STR genotyping, a series of amplifications was performed with templates ranging from 5 to 1000 pg of high-quality genomic DNA. Amplification success was determined by comparing peak heights (in relative fluorescence units) between the DNA and the LNA primers. Peak heights decreased with decreased template amounts for all loci and primers, thus indicating that they were a good approximation for PCR efficiency and reaction success. All primer sets had R^2 correlation values of greater than 0.9 for template amount and peak height. Overall, LNAs were able to increase amplification success for samples containing between 50 and 1000 pg of template DNA. Between these template limits, LNA primers showed an average increase in peak height of 30%, compared to values achieved with standard DNA primers.

Although there was an overall increase in peak heights, there was significant variation among the four loci tested (Fig. 1). One locus (D13S317) showed a decrease in amplification success when LNA primers were used, with LNAs having an average peak height ~98% of the DNA amplifications. The three other loci investigated (D7S820, FGA, and D18S51) had LNA peak heights of 172, 122, and 112% of their corresponding DNA amplifications. At the lower template levels (50 pg and below), the D13S317 locus showed a consistent decrease in amplification success with the LNA primers, while the other three loci were more variable. There were indications that LNAs could increase amplification success in a limited way, although these were not statistically significant. In particular, D7S820 showed the largest increases in peak heights with the use of LNA primers. However, both DNA and LNA amplifications were subject to considerable stochastic processes at these low template levels.

It was noted that incorporation of LNAs into primers gave an increased number of artifacts in the profiles. Stutter was slightly increased in LNA samples, but only by around 5%. At one locus

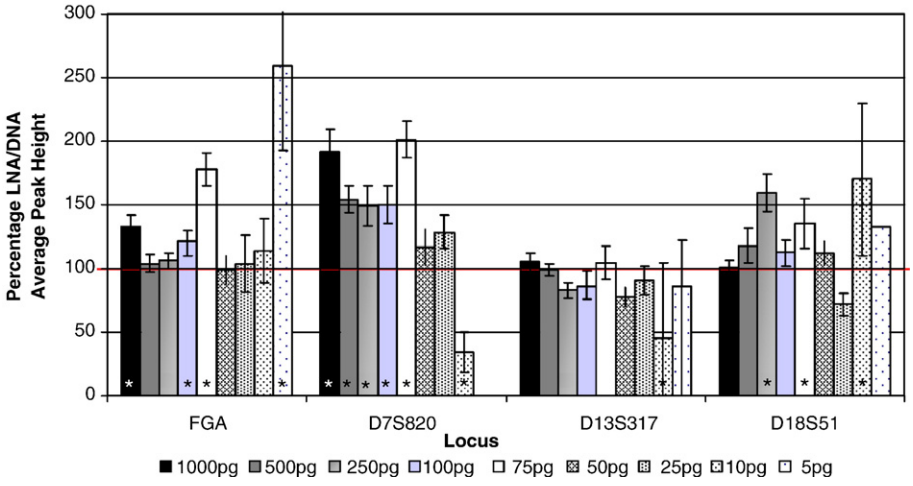

Fig. 1. Average peak height percentages for LNA primers, compared to DNA primers. The result for each template level at each locus is the average of 20 comparisons between DNA and LNA primers. Error bars represent the 95% confidence interval for each average. Significant results (repeated–measures ANOVA, $p<0.05$) are indicated with an asterisk in the base of the column.

(D18S51), the LNA bases appeared to inhibit nontemplate adenylation. The DNA products were consistently pushed to 100% "$n+1$," while around 80% of LNA products remained "n", resulting in split peaks. Increasing or decreasing the length of the 60°C incubation at the end of the cycling protocol failed to reduce the occurrence of the split peaks. Despite the increase in artifacts, both peak height balance and peak area, and balance between alleles within a locus, were increased by the use of LNA bases.

The most successful LNA primer pair (D7S820) showed peak height increases of over 30% compared to the most successful DNA primers (D13S317). Even the least effective LNA primer pair was still 26% better than the least effective DNA primers (D7S820) for average peak heights. This observation suggests that a conventional DNA primer pair that is performing poorly can be significantly improved by the careful incorporation of LNA bases. The more efficient the design of the LNA primers, the greater the increases in peak heights, as suggested by the differences observed in amplification success between the four LNA primers, which had differing numbers and LNA base positions.

Multiplexing LNA primers

To determine if the wider optimal reagent concentration and annealing temperature ranges of the LNA primers were able to simplify the multiplexing process, two duplexes, FGA/D18S51 and D13S317/D7S820, were formed. For the multiplex design, wherever possible an intermediate value of the concentration/temperature for the singleplexes was used. In several instances, the optimal values for the two loci within a multiplex were considerably different—such as for $MgCl_2$ concentration in the FGA/D18S51 DNA duplex-3.5 mM for FGA and 1.2 mM for D18S51. In these situations, the optimal windows for the parameter were examined to determine if there was any overlap. For the DNA primers, the narrower windows (Table 1) proved to be a disadvantage and resulted in the compromised amplification of both loci, due to nonoptimal parameters being used. For the LNA primers, it was possible to select duplex values that lay within the optimal ranges for each locus, thus ensuring that the duplex amplification had the greatest chance of achieving maximum amplification.

For both duplexes, the LNA primers gave increased peak heights—the FGA/D18S51 LNA duplex had an increase of 23%, while the D13S317/D7S820 LNA duplex had an increase of 158% compared to the DNA duplex. The characteristics of each singleplex were carried through to the duplex amplifications—for example, D18S51 still showed the split $n/(n+1)$ peaks in the duplex as in the singleplex amplification. Stutter was slightly increased in the FGA/D18S51 multiplex, which may be a consequence of the increased amplification and peak heights.

However, the D13S317/D7S820 LNA duplex showed substantial numbers of artifacts (Fig. 2). In contrast to the singleplex amplifications, which were relatively clean, this duplex showed greatly increased stutter peaks, which in some samples were larger than the true allele peaks. Attempts were made to reduce the extent of this stutter, including the use of touchdown PCR, reduced cycling time, and reduced numbers of cycles. However, stutter was consistently visible and appeared to be caused by an interaction between the four primers, although none could be predicted using multiplex primer software. Additionally, the increase in stutter was not observed with the corresponding DNA duplex, suggesting that the LNA bases themselves were causing the interaction.

Discussion

The most critical stage of any use of LNAs is the design of the oligonucleotide. Design rules for LNA primers are yet to be formally established; however, several published reports give guidelines regarding their incorporation into PCR primers [5,14,15]. The number and position of the LNA bases used will

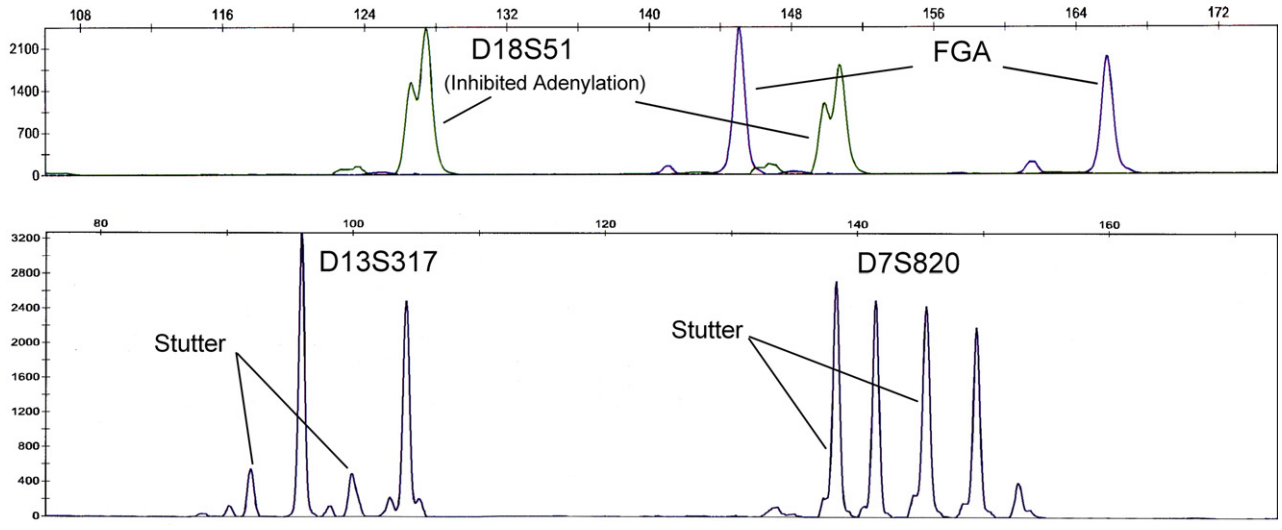

Fig. 2. Electropherograms of LNA duplexes. The top is the FGA/D18S51 duplex, showing no significant artifacts. The bottom is the D13S317/D7S820 duplex. Both loci show increased stutter formation, and D18S51 has a low rate of nontemplate adenylation.

have substantial effects on the outcome of the PCR. Incorporation of too many LNAs can decrease amplification success, as can positioning the LNAs incorrectly, such as at the 3′ end of the primer [5]. There is also a significant difference in the binding strength of different LNA bases, with the change in binding strength order being C > T > G ≫ A, although the contextual sequence can alter binding strength significantly [5,15]. While it may be desirable to increase the binding strength to achieve increased PCR amplification, having a binding strength too high can be detrimental, causing decreased amplification of the target, with an increase in primer–dimers and other artifacts. Therefore, following published design guidelines, our primers had a maximum of three LNA substitutions, and only adenine bases were substituted. Although substituting other LNA bases (e.g., C, T, or G) may further increase amplification success, any such increase in artifact production may outweigh the benefits observed. With adenine–only substitutions, there was no observable increase in the formation of primer–dimers, and no spurious, off-ladder, peaks were seen. The increase in amplification success seen with the LNA primers from 75 pg and greater may be attributed to the higher binding strength of the LNA primers. Having the LNA bases toward the 5′ end of the primer causes 5′ binding strengths that are stronger than the 3′ binding strengths—a common characteristic of high-efficiency primers [5,16]. The presence of the LNA bases also increases target specificity, which may prevent the diversion of the polymerase to off–target binding sites. In this case, peak heights of the targets will be greater, due to the higher rate of polymerase binding and extension.

LNA primers increased the amplification success of both single– and duplex amplifications. In addition, the design and optimization time for multiplexing was considerably reduced compared to multiplexing standard DNA primers. Thus, LNA modifications appear to be suitable for both single– and duplex amplifications of STR loci. The increase in amplification success seen with the duplexes may carry through to multiplexes containing more loci, although primer design issues may become more complex.

The increase in the number of artifacts that accompanies the incorporation of LNA bases is a concern. However, a number of them may be explained by the initial primer design and subsequent increase in binding strength generated by LNAs. The incomplete adenylation of the D18S51 products could be easily remedied with a PIG–tail addition to the reverse primer. The slight increase in stutter seen in the singleplexes may simply be a result of the increased peak heights seen with LNA primers, as the LNA bases within the primers are thought not to have an effect on the stutter formation process. The large increase in stutter, as seen with the D13S317/D7S820 duplex, is less easily explained—however, primer design may be responsible. The D13S317 reverse primer is 4 bases into the repeat region, with imperfect repeats following in the primer sequence. The positioning of the LNA bases suggests that a shift of 4 bp in the primer binding site can occur, and the LNA bases will still be matched with correct template bases. If this occurs with the DNA primers, the incorrectly matched nucleotides between the matching bases may prevent the complete binding and extension of the primer. However, the LNA bases provide much stronger binding and therefore may allow the mismatched primer to bind sufficiently for extension to occur. Therefore, it may be advisable to avoid designing LNA primers that run into repeat regions. However, in situations such as this, the increase in T_m that LNA bases give enables primers to be shortened, which may allow the primer be kept in the same location, but avoid possible mispriming interactions. Examining other loci with difficult priming sites or repeat regions may be beneficial in determining the correct design approach for these primers.

LNAs offer a promising new tool for increasing amplification sensitivity and specificity. While in some cases the increases may be minimal (particularly below 75 pg), in many fields, such as forensic and archaeological genetics, any increase may be beneficial for obtaining a detectable result. It should be noted that a conservative design strategy was used for the LNA primers in this study—it would be possible to increase the binding strength further, and thus possibly the amplification success, by altering the number and placement of LNA bases within the primers. Increased ease of reaction optimization, for both single– and multiplex reactions, also gives LNA an advantage over standard DNA primers. Although care must be taken with the design of LNA primers, published design rules can assist [5,14], with empirical testing adding to current knowledge in this area.

Materials and methods

Primer design and sequences

Four STR loci in common use within the forensic community (FGA, D7S820, D13S317, and D18S51) were selected based on amplification success within commercially available multiplex kits and amplicon target size. These loci have reduced PCR efficiency within multiplexes (compared to the other loci in the multiplex) and have difficult priming sites, with high AT content or imperfect repeat sequences. Primer sequences that have been shown to amplify the target STRs effectively without null alleles or heterozygote peak imbalance were obtained from [17]. LNA primers were designed by the Sigma Proligo Design service, in accordance with design rules suggested by Levin [5]. Sequences remained unchanged between the DNA and the LNA primers—only the LNA bases themselves were altered (Table 2).

Primers were synthesized by Sigma Proligo (NSW, Australia), with forward primers labeled with 5′ phosphoramidite fluorophores (6FAM, HEX, and TET). All primers were HPLC purified.

Table 2
Oligonucleotide primer sequences

Primer name	DNA primer	LNA primer
FGA–F (6FAM)	aaataaaattaggcatatttacaagc	aaatAaaattAggcAtatttacaagc
FGA–R	gctgagtgatttgtctgtaattg	gctgagtgAtttgtctgtaattg
D7S820–F (TET)	gaacacttgtcatagtttagaacgaac	gaacActtgtcatAgtttagaacgaac
D7S820–R	tcattgacagaattgcacca	tcAttgAcagAattgcacca
D13S317–F (TET)	tctgacccatctaacgccta	tctgAcccAtctaacgccta
D13S317–R	cagacagaaagatagatagatgattga	cagacAgaaAgatAgatagatgattga
D18S51–F (HEX)	tgagtgacaaattgagacctt	tgagtgAcaAattgagacctt
D18S51–R	gtcttacaataacagttgctactatt	gtcttAcAataacagttgctactatt

LNA bases are in uppercase.

DNA samples

Ethics approval to collect buccal swabs from informed volunteers was obtained from the La Trobe University ethics committee. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen, VIC, Australia) in accordance with the manufacturer's instructions for the isolation of genomic DNA from swabs. Quantitation was performed with the Quantifiler Human DNA Quantification System (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer, although a commercial DNA sample was used for the standard (K562 High Molecular Weight DNA; Promega, Madison, WI, USA), on an ABI Prism 7500 real-time PCR system (Applied Biosystems).

PCR amplifications

Optimization of PCR reactant concentrations and annealing temperatures was performed using a Taguchi factorial array design [9,10]. Amplifications were performed in 25-μl volumes, containing 1× FastStart PCR buffer (Roche, Mannheim, Germany), 200 μmol/L deoxynucleotide triphosphates (Roche; dATP, dCTP, dGTP, dTTP), empirically adjusted $MgCl_2$ and primer concentrations, and 1 U of Roche FastStart Taq polymerase. Thermal cycling was performed in a GeneAmp 9700 (Applied Biosystems) using the following conditions: 95°C for 10 min; 28 cycles of 94°C for 1 min, variable annealing temperatures for 1 min, and 72°C for 1 min; followed by 45 min at 60°C. Annealing temperatures were determined empirically for each primer pair.

Analysis on ABI 3100 genetic analyzer

Samples were prepared with 12 μl of Hi–Di formamide (Applied Biosystems), 0.1 μl GS400 size standard labeled with ROX, and 1 μl PCR product. Samples were denatured and snap–cooled prior to injection on the ABI 3100 genetic analyzer. Default run parameters for the POP–4 36-cm fragment analysis module were used—electrokinetic injection at 3 kV for 10 s, with separation occurring at 15 kV for 30 min at a 60°C run temperature. Raw data were genotyped with GeneMapper ID version 3.01 (Applied Biosystems). Statistical analysis was performed with SPSS version 12.0.1, using repeated-measures ANOVA.

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