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Research article

Locked nucleic acids: Increased trace DNA amplification success with improved primers

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Abstract

Locked nucleic acids (LNAs) are a conformationally restricted DNA analog, which can be incorporated into oligonucleotides to increase binding strength. To investigate if LNAs increase amplification success for trace DNA samples in a forensic context, primer sequences for four routinely used STR loci (FGA, D7S820, D13S317 and D18S51) have been altered to include LNA bases. The LNA modified primers display a broader tolerance to a range of reaction conditions compared to unmodified DNA primers, with higher T_ms giving increased specificity. Increased peak heights, improved peak height ratios and decreased template requirements were seen with LNA primers. The increased amplification success of LNA primers, and broader range of optimal reaction conditions, suggest that using LNA primers for multiplex STR genotyping assays could be highly beneficial for trace DNA genotyping.

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

1. Introduction

Locked nucleic acids (LNAs) are a novel nucleic acid analog, and are a promising tool for increasing DNA amplification success. The incorporation of LNA nucleotides into real-time PCR probes and primers has been shown to decrease Ct values significantly [1,2] and decrease minimum template requirements, with a corresponding increase in amplification efficiency [3]. The improvement in amplification success using LNA oligonucleotides suggests that they may also be beneficial for standard PCR. We have compared modified primers containing LNA bases with their standard DNA primer counterparts for amplification sensitivity and success at low template levels, and examined the multiplexing ability of each primer pair, to determine if the LNA modifications are suitable for PCR primers used in DNA STR profiling.

2. Materials and methods

2.1. Primer sequences

Primer sequences were obtained from [4]. LNA primers (Table 1) were designed by the Sigma Proligo Design service, in accordance with design rules suggested by Levin [3] and Latorra [5]. Primers were synthesised and HPLC purified by Sigma, with forward primers labelled with 5′ phosphoramidite fluorophores.

2.2. DNA samples

DNA was extracted using the QIAamp DNA Micro kit (Qiagen) in accordance with the manufacturer's instructions, and quantitated with the Quantifiler Human DNA Quantification System (Applied Biosystems) on a ABI Prism 7500 real-time PCR System (Applied Biosystems).

2.3. PCR amplifications and electrophoresis

Optimisation of PCR reactant concentrations temperatures was performed using a Taguchi factorial array design [6].

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Table 1
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 in capitals.

Amplifications were performed in 25 μL volumes, containing 1× FastStart PCR buffer (Roche), 200 μmol/L dNTPs (Roche) 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) with 95 °C for 10 min, 28 cycles of 94 °C for 1 min, annealing 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.

Samples were analysed with a ABI3100 Genetic Analyser, with Hi-Di formamide (Applied Biosystems) and GS400 size standard (Applied Biosystems). Default run parameters for the POP-4 36 cm fragment analysis module were used. Raw data were genotyped with GeneMapper ID v 3.01 (Applied Biosystems).

3. Results and discussion

3.1. Low copy number LNA amplifications

LNA increased 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 standard DNA primers, although variation was noted between the four loci (Table 2). At very low template levels, both the DNA and LNA amplifications were subject to considerable stochastic variation, preventing accurate comparisons between the two primer types. However, there were indications that LNA could improve trace profiling at some loci. Overall, 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 was still 26% better than the least effective DNA primer (D7S820) for average peak heights. These findings suggest that a conventional DNA primer which is performing poorly can be significantly improved by careful incorporation of LNA bases. We applied the LNA design rules [3,5] in a conservative manner to our LNA primers. Alternative primer designs may yield even better outcomes.

There was a slight increase in the number of artefacts in the LNA amplifications. Stutter was slightly elevated, but only by around 5%. At D18S51, the LNA bases appeared to inhibit non-template adenylation, with 80% of LNA products remaining unadenylated. However, peak height and peak area balance between alleles was increased by the use of LNA bases.

Table 2
Average peak height percentages for LNA primers, compared to DNA primers

Template amount (pg)	Locus				
	FGA	D7S820	D13S317	D18S51	Average
1000	***132***	***192***	105	101	***128***
500	104	***154***	99	118	***120***
250	106	***149***	83	***159***	***123***
100	***121***	***150***	87	113	112
75	***177***	***201***	105	***135***	***150***
50	99	117	78	112	108
25	104	128	91	72	100
10	114	34	***45***	***170***	94
5	***259***	–	86	–	***132***

Bold italic type indicates a significant difference ($p < 0.05$) between LNA primers and standard DNA primers. Results are averages of 20 comparisons between DNA and LNA, at each locus for each template amount.

3.2. Multiplexing LNA primers

During the optimisation of the primer pairs, it was noted that the LNA primers had wider optimal ranges for annealing temperature, and $MgCl_2$ and primer concentrations. These ranges proved beneficial for creating duplex amplifications with the LNA primers, with less adjustment of parameters required than for the DNA primers. LNA primers gave increased amplification success with the two duplexes, FGA/D18S51 (an increase of 23% in peak height), and D13S317/D7S820 (increase of 158%). However, the D13S317/D7S820 LNA duplex showed greatly increased stutter with the LNA primers only. This appeared to be caused by an interaction between the four primers, although none was predicted using multiplex primer software. The increase in stutter was not observed with the corresponding DNA duplex, suggesting that the LNA bases themselves were causing the interaction.

4. Conclusions

LNA offers a promising new tool for increasing amplification sensitivity and specificity. LNA amplifications showed higher peak heights compared to standard DNA amplifications, with greater success at template levels as low as 50 pg. Increased ease of reaction optimisation, for both single and

multiplex reactions, also give LNA primers an advantage over standard DNA primers. It appears that the design of the LNA primers is crucial, and although published design rules can assist [3], empirical testing of each primer design is necessary. Overall, LNA could be beneficial in modifying existing PCR primers for the amplification of STR loci in current multiplex systems.

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Conflict of interest

None.

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