Increased amplification success from forensic samples with locked nucleic acids

Kaye N. Ballantyne a, b,*, Roland A.H. van Oorschot a, R. John Mitchell b

a Biology Division, Victoria Police Forensic Services Department, Macleod, Victoria, Australia
b Genetics Department, La Trobe University, Bundoora, Victoria, Australia

A R T I C L E   I N F O

Article history:
Received 28 January 2010
Received in revised form 16 March 2010
Accepted 7 April 2010

Keywords:
Locked nucleic acids
Amplification
Trace DNA
Forensic

A B S T R A C T

Inadequate sample quantities and qualities can commonly result in poor DNA amplification success rates for forensic case samples. In some instances, modifying the PCR protocol or components may assist profiling by overcoming inhibition, or reducing the threshold required for successful amplification and detection. Incorporation of locked nucleic acids (LNAs) into PCR primers has previously been shown to increase amplification success for a range of non-forensic sample types and applications. To investigate their use in a forensic context, the PCR primers for four commonly used STR loci have been redesigned to include LNA bases. The modified LNA primers provided significantly increased amplification success when compared to standard DNA primers, with both high-quality buccal samples and simulated forensic casework samples. Peak heights increased by as much as 5.75 × for the singleplex amplifications. When incorporated into multiplexes, the LNA primers continued to outperform standard DNA primers, with increased ease of optimisation, and increased amplification success. The use of LNAs in PCR primers can greatly assist the profiling of a range of samples, and increase success rates from challenging forensic samples.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

There are many challenges involved with the amplification of crime-scene samples for forensic STR profiling. Samples may contain inhibitory substance, be heavily degraded, have only trace quantities of biological material, or be from multiple donors. From these often minute quantities of cellular material, analysts attempt to obtain results from 9 or more STR loci. Unsurprisingly, many samples may give inadequate results. Partial profiles, amplification bias and allele drop-in can all be observed with compromised samples [1]. While current multiplexes perform exceptionally well with a wide range of samples, new methods and technologies are being developed which could further increase the success rates of current STR profiling reactions from compromised samples.

One approach for increasing the amplification success rate is to alter the reaction components and parameters of the PCR. While considerable research has focused on adjusting cycling times and numbers, and reagent concentrations (for examples see [1–3]), one avenue that has remained largely unexplored is the adjustment of the STR primers themselves. In general, primers for forensic profiling are designed to minimise the amplicon size (miniSTRs [4]), to maximise multiplexing potential, and/or to produce a highly specific product [5]. However, analysis of published primer sequences and their binding sites reveals that often these primers are located in ‘difficult’ sites, with high AT content, imperfect repeats, or homopolymer stretches. These features can lead to decreased priming efficiency, resulting in lower PCR product formation rates than might otherwise be achieved.

Increasing primer binding and extension rates can be difficult, particularly without changing the primer sequence. A novel synthetic nucleic acid, called Locked Nucleic Acid (LNA) has been shown to increase oligonucleotide binding strength and affinity for several applications, including fluorescence polarisation, SNP probe based genotyping, in situ hybridisation and real-time PCR ([6–8] and reviews [9–11]). There are many benefits to using LNAs in PCR primers to increase amplification sensitivity and success. First, they have the highest binding strength of any nucleic acid analog discovered to date, giving higher thermal stability than either DNA or RNA [12,13]. This gives increased duplex stability, and greatly increased mismatch discrimination [14,15]. Second, LNA bases can be easily incorporated into existing primer sequences – there is no need to alter the priming site or sequence. A novel synthetic nucleic acid, called Locked Nucleic Acid (LNA) has been shown to increase oligonucleotide binding strength and affinity for several applications, including fluorescence polarisation, SNP probe based genotyping, in situ hybridisation and real-time PCR ([6–8] and reviews [9–11]). There are many benefits to using LNAs in PCR primers to increase amplification sensitivity and success. First, they have the highest binding strength of any nucleic acid analog discovered to date, giving higher thermal stability than either DNA or RNA [12,13]. This gives increased duplex stability, and greatly increased mismatch discrimination [14,15]. Second, LNA bases can be easily incorporated into existing primer sequences – there is no need to alter the priming site or sequence. Third, the increased binding strength and duplex stability increase the Tm of oligonucleotides [16]. This allows either the PCR to be conducted at a higher temperature, giving increased specificity, or primers and probes to be shortened.

Previous studies have shown that the use of LNAs in PCR primers can improve results, giving increased product formation, decreased
mispriming, and improve poorly performing primers [6,15,17]. We have previously incorporated LNA nucleotides into existing forensically relevant miniSTR primer sequences to examine if they can increase genotyping success for low copy number samples. Initial results with high-quality buccal cell DNA indicated that LNAs could increase the success rate of amplifications by an average of 1.24× (LNA average peak height/DNA average peak height). The greatest increases were seen with template amounts above 50 pg, although there were clear indications that LNAs could assist with a range of sample concentrations [18].

As the initial investigations indicated that LNAs increased amplification success, we have continued the investigations with forensically relevant sample types, and have also performed some preliminary examinations of the ability of the LNA primers to be multiplexed. Results show that LNAs could be a useful tool for forensic molecular biology, and indeed molecular genetics as a whole to aid with genotyping of a wide range of sample types.

2. Methods and materials

2.1. Primer design and sequences

Four STR loci (FGA, D7S820, D13S317 and D18S51) were selected from commonly used forensic multiplexes, which had either lower amplification success rates (peak heights, allelic dropout rates), and/or compromised priming sites (imperfect repeats or high AT compositions). Primer sequences were obtained from [4], which have been shown to amplify the target loci efficiently, without null alleles or heterozygote peak imbalance. LNA primers were designed with assistance from the Sigma Proligo design service (www.sigma-aldrich.com), in accordance with design guidelines put forward by Levin et al. [15] and Latorra et al. [19]. The nucleotide sequence of the primers remained unchanged – the only difference between the two types of primers was the substitution of 1–3 LNA nucleotides for the corresponding DNA nucleotides (Table 1). A conservative design strategy was employed – only adenine bases were converted to LNA (adenine being the weakest binding of the LNAs), and a maximum of 3 LNAs were added to each primer. Additionally, the LNAs were predominantly added to the 5′ regions of the primers – Levin et al. [15] have suggested that this design strategy gives increased specificity and amplification, compared to 3′ incorporation of LNA. Primers were synthesised by Sigma Proligo (NSW, Australia), with forward primers labelled with a 5′ phosphoramidite fluorophore (6FAM, HEX or TET). All primers were HPLC purified.

2.2. DNA samples and extraction

Ethics approval was given by the La Trobe University ethics committee to obtain DNA samples from informed volunteers. Buccal cell DNA was extracted using the QIAamp DNA Micro kit (Qiagen, Victoria, Australia) in accordance with the manufacturer’s instructions for isolation of genomic DNA from swabs. Simulated casework samples (n = 20) were extracted with the DNA IQ system (Promega, Madison, WI), using the small sample casework protocol as recommended by the manufacturer. Quantitation was performed with the Quantifiler Human DNA Quantification System (Applied Biosystems, Foster City, CA) as recommended by the manufacturer, although a commercial DNA sample (K562 High Molecular Weight DNA, Promega, Madison, WI) was used for the standard as recommended by Koukoulas et al. [20], on an ABI Prism 7500 real-time PCR System (Applied Biosystems).

2.3. Amplification

Each primer pair was optimised separately, using a Taguchi experimental design method [21]. This approach allowed the precise determination of the optimal concentration of primers and MgCl₂, and annealing temperature. Amplifications were performed in 25 μl volumes, containing 1× FastStart PCR buffer (Roche, Mannheim, Germany), 200 μmol/l deoxynucleotide triphosphates (Roche, dNTPs; dATP, dCTP, dGTP, dTTP), empirically adjusted MgCl₂ and primer concentrations, and 1 U of Roche FastStart Taq polymerase. Thermal cycling was performed in a GeneAmp 9700 (Applied Biosystems) using: 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. Profiler Plus (AmpFISTR™ Profiler Plus, Applied Biosystems) multiplex genotyping reactions were performed as recommended by the manufacturer. Every reaction was performed in duplicate. For every amplification performed for comparison purposes, DNA concentration, cycle number and analysis conditions were kept constant between DNA and LNA amplifications. To ensure that each primer pair was performing optimally however, MgCl₂, primer concentrations and annealing temperatures were varied in accordance with the primer requirements.

2.4. Capillary electrophoresis, genotyping and statistics

Samples were prepared with 12 μl of Hi-Di formamide (Applied Biosystems), 0.1 μl GS400 size standard, labelled with ROX, and with 1 μl PCR product. Samples were denatured and snap-cooled prior to injection on the ABI3100 Genetic Analyser. Default run parameters for the POP-4 36 cm fragment analysis module were used – electrophoretic 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 v3.01 (Applied Biosystems). Statistical calculations were performed with the Analyse-It plug-in for Microsoft Excel.

3. Results

3.1. Amplification of simulated casework samples with LNA primers

The success of the LNA primers with high-quality DNA [19] suggested that the modified primers may be useful for forensic casework samples. Therefore, a number of casework type samples (including airmail stickers (paper labels with adhesive backing, 2 samples), bloodstains (3), bone (2), cigarette butts (3), hair (3), vaginal swabs containing sperm (2 swabs, giving 2 epithelial fractions and 2 sperm fractions post-extraction), and touched items (3)) were selected and extracted. There was a large range in concentration, from 52 pg/μl to 3.32 ng/μl. 1 μl of each sample was amplified for each locus with both the DNA and LNA primer sets, in duplicate, for a total of 80 amplifications per locus (320...
Average DNA/LNA ratios and concentrations for each sample are presented in Supplementary Table 1.

Although there were differences among sample types, and among the 4 loci, overall the LNA primers showed a considerable increase in peak heights (and peak areas, which showed the same patterns) compared to the standard DNA primers, with LNA peak heights on average 1.69 × greater than corresponding DNA peak heights for the same locus (calculated as LNA peak height/DNA peak height, and averaged across alleles and duplicate amplifications). Of the 4 loci, D18S51 showed the largest increases, with an average increase of 2.64 in peak height, followed by D7S820, with 2.09. There was considerable variation in success between the different sample types. Absolute peak height correlated strongly with template amount ($R^2 > 0.93$ for all loci), for both DNA and LNA when examined individually, as expected. However, there was no correlation seen between the template amount and the difference in peak heights observed between DNA and LNA primers – the inclusion of LNA into the primers resulted in the same percentage increase in peak height regardless of whether 50 pg or 3 ng was added (Fig. 1).

The touched item samples, representing trace DNA, showed the largest increase using LNA primers across all loci (Fig. 2). Average peak height increases ranged from 1.46 × (D13S317, an average increase of 650 RFU), to 5.75 × (D7S820, average increase of 3000 RFU). Other sample categories that showed large increases for the LNA primers were cigarette butts, and seminal fraction of differential extractions. The only sample type to show a consistent decrease was extracts from bone. These were predominately negative for both DNA and LNA, and only had very low peak heights (<100 RFU) for those samples that were successfully amplified. This failure to amplify may be due to a non-optimal extraction procedure, resulting in the presence of inhibitors.

The LNA amplifications were also compared to the full 10 locus Profiler Plus amplifications. All comparisons between Profiler Plus, DNA and LNA amplifications involved the same loci, same template amounts and the same number of cycles. Although absolute peak heights cannot be compared between single- and multi-plexes, and the different primer sequences, the comparison gives an indication of how the LNAs are performing, compared to the standard methodology. LNA peak heights were, on average, 2.88 × greater than the Profiler Plus peak heights (comparison between 320 LNA and 320 Profiler Plus amplifications). In comparison, the DNA miniSTR primers used in the present study, gave peak heights that were only 1.56 × greater. A caveat on this finding is that there were differences in the amplification of the sample types between the LNA and DNA primers, and the Profiler Plus multiplex. Sample types that contain potential inhibitors, such as blood and cigarette butts, performed poorly with the singleplex amplifications, compared to Profiler Plus. This could be due to the lack of bovine serum albumin (BSA) in the singleplex reactions, which has been shown to alleviate inhibition. However, this does not invalidate the DNA to LNA comparisons, as neither amplification type contained BSA.

There was a small increase in the number of artefacts in the LNA profiles. Stutter was slightly increased (~5% increase in peak height) at two of the loci (D7S820 and D13S317), and non-template addition (adenylation) was partially inhibited in D18S51 LNA samples. While increased stutter may be partially explained by the higher peak heights, it appears that the design of the primers themselves affected the formation of stutter, as 2 of the 4 LNA primers did not show any increase in stutter. Artefact production was independent of sample origin. Another factor to consider in the use of LNA primers is the slight change in molecular weight caused by the LNA bases. Increases in size (bp) ranged from 0.11 to 0.39. Although this is within the 0.5 bp window allowed in GeneMapper ID for allelic designation, the consistent increase within each primer pair suggests that it is advisable to create allelic ladders and macros using LNA primers with the same number of substituted bases, particularly if primers contain more LNAs than described here.

### 3.2. Multiplexing LNA primers

Any primer set to be used for forensic purposes must be capable of being multiplexed. The multiplexing ability of LNA primers was
examined to determine if the stronger binding of the LNA bases caused increased inter-primer interaction. Two duplexes were developed, FGA/D18S51 and D7S820/D13S317. In general, the characteristics observed with the singleplexes were carried through to the duplexes, with significantly increased peak heights and amplification success with the LNA duplexes compared to the DNA duplexes. The D7/D13 duplex did exhibit increased stutter, as noted previously. Duplex optimisation proved a lot simpler task for the LNA primers than the DNA primers, due to the wider range of conditions under which LNA primers operate optimally, particularly temperature (average optimal window for LNA is 5.75°C), and MgCl₂ concentration (average window LNA 0.625 mM, DNA 0.5 mM). This advantage reduced the amount of time, and number of reactions, required to optimise the reactions to obtain maximum peak height and balance. Amplification results were similar to the singleplex results, with a slight increase in the fold increase in peak height compared to the singleplexes. The FGA/D18S51 showed an average increase of 0.43 fold over the corresponding singleplex averages (2.18× increase, compared to 1.75), and the D7S820/D13S317 multiplex had a 0.22× increase (1.87×, compared to 1.65). Thus, not only is multiplexing substantially easier with LNA primers, but results are also enhanced compared to DNA primer multiplexes.

4. Discussion

The incorporation of locked nucleic acids in PCR primers proved beneficial for amplifying a wide range of samples for STR genotyping. There were significant improvements in peak heights and amplification success for both high-quality buccal DNA and a range of forensic-type samples. While there were differences among the four loci in the LNA primers' ability to amplify, and differences in efficiency between template levels, overall there was a positive improvement in amplification outcomes when LNAs were used. Although small sample numbers were used in this investigative study, the increases observed with the DNA primers were high enough to be statistically significant in many cases. It was noted that the simulated casework samples gave higher percentage increases in peak height for LNA when compared to the standard buccal samples examined in a previous study [18]. This may be due to the difference in extraction methods – buccal DNA was extracted using a QIAamp method (Qiagen), while ‘casework’ samples were extracted with DNA IQ (Promega). In our laboratory DNA IQ extracted samples frequently give increased peak heights, and appear to amplify better than Chelex or QIAamp extracted samples (A. Poy, personal communication). Thus, a slight change in the extraction buffer seems to result in improved amplification, particularly for the LNA primers, which require different reagent concentrations to those for standard DNA primers. It has previously been observed that LNA-modified duplexes have different rates of both water and counterion uptakes than standard duplexes, factors which may explain why the performance of the LNA increased even more with the IQ samples [22]. The alteration of the PCR buffer may also further assist the amplification, particularly the addition of BSA or a similar molecular crowder [23] to overcome inhibition, and promote unbiased amplification.

The differences observed among the four loci may be due to the slightly different LNA positioning within each primer. McGue et al. [24] noted that the neighbouring DNA bases altered the binding strength of each LNA base, and that with increasing amounts of modification the entire nucleic acid structure changes to a B-type helix [25]. The introduction of too many modifications, bringing with them a radical change in helix conformation, can, however, be detrimental to amplification [19], possibly due to the inability of the primer to dissociate from the template following extension. Therefore, it is critical to design any LNA-modified primers carefully, with reference to both published design guidelines and empirical studies. A very conservative design strategy was employed here, and it is worth noting that these results could be viewed as evidence of the ‘minimum’ effect of LNAs in PCR primers.

However, as there are yet no firm ‘rules’ for LNA design, it can be difficult to predict how the LNA bases will respond. We observed a number of artefacts, caused by the LNA bases, which were not predicted from the current literature on LNAs. The inhibition of adenylation with the D18S51 LNA primers cannot be readily explained – the positioning of the LNAs should not affect the polymerase action. However, this may be remedied by PIG-tailing (adding a GT tail) to the primer [26]. The increased stutter observed, particularly with the D7S820/D13S317 duplex, was of more concern as it may cause incorrect genotype/mixture assignment. However, it appears that this is predominantly caused by the positioning of the primers partially within the STR sequence itself. Both forward primers have full or partial repeat motifs at the 3' end. It is possible that the increased binding strength of the LNAs allows the primer to shift along the template by 4–8 bases, and the altered duplex conformation of the LNA primer/template tolerates slight mismatches. Therefore, to avoid this possibility, it is recommended that any primers containing LNA bases be situated outside repeat regions.

Unfortunately, neither a clear pattern has emerged from the current preliminary investigation regarding the best overall design strategy, nor why one primer pair in particular (D13S317) did not perform as well as the others. No correlation was found between the spacing of the LNA bases, the surrounding sequence, or the level of secondary structure within the primer which might
account for the differences (data not shown). Further, little new research has emerged in the years since Latorra et al. [19] published the most detailed guide to the placement of LNA bases within PCR primers. Thorough and exhaustive studies are still required to elucidate the optimal placement strategies, although the general guidelines are becoming clearer with each study performed using LNAs.

Overall the LNA modifications appear to be a promising new area of research for improving the amplification of forensic samples. The increases observed in peak heights, combined with the ease of optimising and duplexing the primers, suggest that they may be valuable for future PCR assays within the forensic profiling field.

Acknowledgments

The authors are grateful to the Australian Research Council and Victoria Police Forensic Services Department for providing funding for the study, and to the Sigma Proligo design team for invaluable help and advice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2010.04.001.

References