Redesigning Quantification: An LNA-Enhanced qPCR Assay for Highly Degraded Forensic Samples

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INTRODUCTION

DNA quantification is a crucial step in the forensic workflow to assess sample quantity and quality and determine suitability for downstream analysis. Conventional probe-based qPCR assays typically target regions of 80-90 bp, which can underestimate the amount of amplifiable DNA in highly degraded samples [1]. Although intercalating dye-based assays can amplify shorter fragments, they often overestimate total DNA due to non-specific dye binding. These limitations pose challenges for sequencing-based approaches that can work with shorter DNA fragments.

This study presents the development of a novel qPCR assay targeting a short autosomal fragment (<60 bp) specifically designed for compromised forensic samples intended for whole-genome sequencing (WGS), where the input requirements are different [2]. The assay incorporates locked nucleic acids (LNAs) into both primers and probe to enhance binding affinity and specificity while minimizing amplicon length [3]. This represents the first forensic qPCR assay to utilize short LNA probes and evaluates the feasibility of combining LNA probes for qPCR, supporting WGS workflows through a more informative pre-screening approach for compromised forensic samples.

MATERIALS & METHODS

a) Identifying a target region

A multicopy region was selected to increase the signal strength during the detection process. RNU2-1, a 188bp non protein coding, multi copy gene located in chromosome 17 was selected as the target region for this assay.

b) Primer and probe design

Primers were designed using NCBI BLAST and modified with LNAs via IDT software (IDT Technologies, Coralville, Iowa); GC content kept below 60%, LNAs avoided at the 3′ end, and Tm set to ~64 °C

A PrimeTime® probe designed using the IDT software with LNA's positioned to make Tm ~6 °C higher than primer Tm . Cy5 was used as the reporter dye with Iowa Black as the quencher

Primer and probe specificity verified via Blast database search; dimer formation checked using manual and using an online Multiple Primer Analyzer Tool (Thermofisher Scientific, Waltham, USA)

c) Assessment of primer and probe performance

A gradient PCR was performed (+/- 10% Tm at 60 °C) on an Eppendorf Mastercyler Gradient PCR Thermal Cycler (Model 5331) (Eppendorf, Hamburg, Germany) using the primer pair of standard-length following manufacturer's protocols to confirm that the selected target locus was the sole region amplified. Gel electrophoresis (2% Agarose) was carried out for the PCR products.

Standard and LNA-modified primers and probes were then tested at various concentrations (Table 01) in TaqMan (Thermofisher Scientific) qPCR reactions using a DNA standard in a 5 x 10x dilution series (25ng – 0.002ng/µl) on a QuantStudio 5 (Thermofisher Scientific) according to manufacturer's protocols. Standard curves from these reactions were analyzed by calculating efficiency and R^2 values to determine optimum primer-probe concentrations.

To assess fragment distribution, a subset of qPCR reactions was analyzed on a QIAxcel (QIAGEN, Hilden, Germany) using a QIAxcel DNA High Sensitivity Assay (QIAGEN).

RESULTS & DISCUSSION

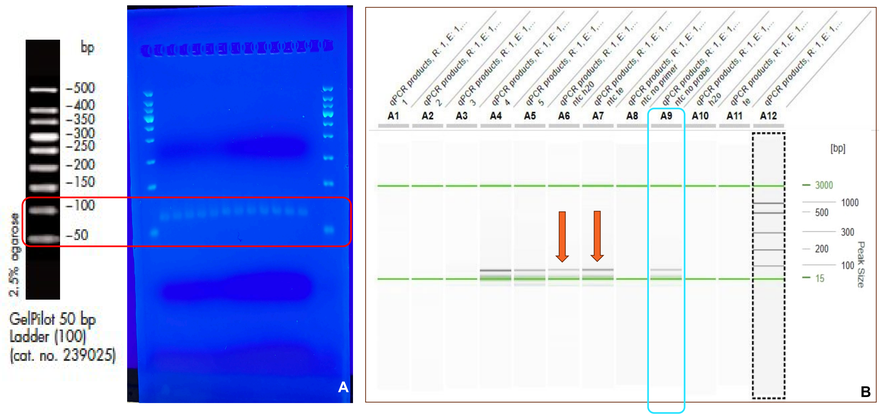

Figure 01: *(A) Evaluation of the success of the target region by gel electrophoresis following gradient PCR. The amplifies band is sized at ~ 60bp using the 50bp size ladder (GelPilot 50 bp Ladder, QIAGEN), indicated in the red box.* ***(B)*** *Fragment analysis using the QIAxcel following qPCR. Lanes A1 through A5: diluted DNA standards A1: 25ng/uL, A2: 2.5ng/uL both diluted twenty-fold, A3: 0.25ng/uL diluted fivefold all diluted prior to the run, A4: 0.025ng/uL and A5: 0.0025ng/uL were added undiluted into the tube. A total volume of 6uL was added to each tube. A6: NTC with water, A7: NTC with TE^{-4} (both indicated with orange arrows indicating fragments in the NTC's), A8: NTC without added primer, A9: NTC without added probe (indicated in the blue box), A10: Millipore water used for the dilution series, A11: TE^{-4} used for the reconstitution of the primers and probe and A12: the 100bp-1kb ladder (QX DNA HS Size Marker) (QIAGEN).*

Determining the optimal primer and probe concentrations

Primer pair	Probe concentration (nM)	Primer concentration (nM)	R^2 value	Efficiency	Amplicon size
FWD GAGCTTGCTCCGTCCACTCC REV GTGCACCGTTCCTGGAGGT	50	200	0.996	105.251	62bp
		300	0.996	102.831	
		400	0.998	107.885	
	100	200	0.998	102.972	
		300	0.999	105.630	
		400	0.995	108.964	
	200	200	0.999	90.410	
	300	200	0.998	84.982	
	500	200	0.999	80.815	
FWD GAG CTT GCT C+CG TCC ACT C REV GTG CAC CGT T+CC TGG AGG	100	200	1.000	96.728	62bp
		300	0.996	103.336	
		400	0.996	101.970	
FWD CTT GCT CCG T+CC ACT CC REV GCA CCG TTC +CTG GAG G	50	50	0.999	68.920	57bp
		100	0.999	88.197	
	100	100	0.999	86.037	
		200	0.999	94.855	
		300	0.999	95.584	
		400	0.996	105.635	
	100 (without BSA)	400	1.000	104.296	

Table 01*: Primer and probe concentrations with efficiencies and R^2 values for each combination*

Standard-length primers (Figure 02 A) were first used to confirm that the selected target locus was the sole region amplified. Gel electrophoresis of the PCR products showed a single, distinct band near the 50 bp marker (Figure 01). Two additional primer sets were designed by a) introducing locked nucleic acid bases and b) reducing overall primer length to generate shorter amplicons (Figure 02, B and C).

A very short primer set containing more than four LNA modifications failed to produce amplification, likely due to polymerase inhibition caused by excessive structural rigidity at the 3' end. Optimization experiments with the remaining primer sets explored a range of primer and probe concentrations. A decrease in amplification efficiency was observed at higher probe concentrations across all primer pairs. Among them, the standard-length primers consistently demonstrated the highest overall efficiency at both 50 nM and 100 nM probe concentrations. Nevertheless, considering the study's objective to minimize amplicon length, the trimmed primer pair containing a single LNA incorporation was selected as the final design. Optimal amplification was achieved using 400 nM primers and 100 nM probe (Table 01, highlighted in blue).

All primer–probe combinations showed amplification in the no-template controls (NTCs), but only at high cycle threshold (Ct) values (Figure 03), suggesting minimal contamination or nonspecific activity. To further investigate these observations, a subset of qPCR reactions and control conditions including blanks containing H_2O or low TE buffer, reactions with probe but no primers, and reactions with primers but no probe were analyzed using the QIAxcel system (QIAGEN) (Figure 01B). Unexpected bands were detected in both the water and low TE blanks, as well as in the primer–no-probe reaction blank. These findings suggest potential low-level contamination or secondary structure formation within the LNA-modified oligonucleotides, possibly resulting from their enhanced binding affinity and structural stability. Additional analyses are required to elucidate the underlying cause.

A) PRIMER PAIR OF STANDARD LENGTH - 62bp

AATGTGGATGAGAGTGGGACGGTGACGGCGGGCGCGAAGGCGAGCGCATCGCTTCTCGGC
CTTTTGGCTA AGATCAAGTGTAGTATCTGTTCTTATCAGTTTAATATCTGATACGTCCTCTATCCG
AGGACAATATATTA AATGGATTTTTGGAGCAGGGAGATGGAATAGGAGCTTGCTCCGTCCACTC
CACGCATCGACCTGGTATTGCAGTACCTCCAGGAACGGTGCACCCCCTCCGGGGATACAACG
TGTTTCCTAAAA

B) PRIMER PAIR OF STANDARD LENGTH –1bp FROM 5' END WITH 1 LNA ADDED - 62bp

AATGTGGATGAGAGTGGGACGGTGACGGCGGGCGCGAAGGCGAGCGCATCGCTTCTCGGC
CTTTTGGCTA AGATCAAGTGTAGTATCTGTTCTTATCAGTTTAATATCTGATACGTCCTCTATCCG
AGGACAATATATTA AATGGATTTTTGGAGCAGGGAGATGGAATAGGAGCTTGCTCCGTCCACTC
CACGCATCGACCTGGTATTGCAGTACCTCCAGGAACGGTGCACCCCCTCCGGGGATACAACG
TGTTTCCTAAAA

C) TRIMMED PRIMER PAIR WITH 1 LNA ADDED - 57bp

AATGTGGATGAGAGTGGGACGGTGACGGCGGGCGCGAAGGCGAGCGCATCGCTTCTCGGC
CTTTTGGCTA AGATCAAGTGTAGTATCTGTTCTTATCAGTTTAATATCTGATACGTCCTCTATCCG
AGGACAATATATTA AATGGATTTTTGGAGCAGGGAGATGGAATAGGAGCTTGCTCCGTCCACTC
CACGCATCGACCTGGTATTGCAGTACCTCCAGGAACGGTGCACCCCCTCCGGGGATACAACG
TGTTTCCTAAAA

Figure 02*: Primer pairs and probe designed for the RNU2-1 target region. The location of the forward primers are indicated in yellow, the reverse primers in blue, and the probe in pink, and the LNA nucleotide substitutions in red within the primers and the probe.*

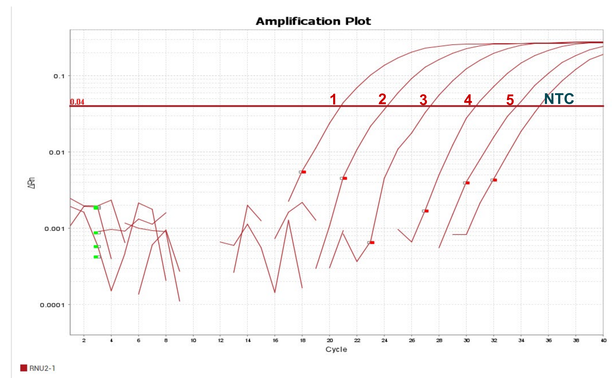

Figure 03*: The amplification plot for standard DNA concentrations of 25ng/uL, 2.5ng/uL, 0.25ng/uL, 0.025ng/uL, and 0.0025ng/uL as standards 1 through 5 respectively at 100nM and 400nM probe and primer concentrations. The NTC showed amplification at later cycles.*

CONCLUSIONS

- ❑ This work represents the first forensic application of LNA-modified oligonucleotides.
- ❑ LNA-modified primers provide an effective strategy for amplifying short DNA fragments.
- ❑ Determining the appropriate number of LNA incorporations for efficient amplification proved challenging, as excessive LNA content can hinder polymerase activity.
- ❑ Further optimization studies investigating both the number and positional placement of LNA bases within the primer sequences are needed to refine primer design and improve qPCR assay performance.

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ACKNOWLEDGEMENTS

This research was partially funded by an internal research grant from Sam Houston State University.

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