

# Discrimination of Alternative Spliced Isoforms by Real-Time PCR Using Locked Nucleic Acid (LNA) Substituted Primer

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**Abstract**—Determination of quantitative expression levels of alternatively spliced isoforms provides an important approach to the understanding of the functional significance of each isoform. Real-time PCR using exon junction overlapping primers has been shown to allow specific detection of each isoform. However, this design often suffers from severe cross amplification of sequences with high homology at the exon junctions. We used human GFR $\alpha$ 2b as a model to evaluate the specificity of primers substituted with locked nucleic acids (LNAs). We demonstrate here that single LNA substitutions at different positions of 3' terminus could improve the discrimination of the primers against GFR $\alpha$ 2a template, a highly homologous isoform. While LNA substitutions of GFR $\alpha$ 2b primer at the residues possessing different sequences as GFR $\alpha$ 2a has limited improvement in specificity, two consecutive LNA substitutions preceding the different sequences has dramatically improved the discrimination by greater than 100,000-fold compared to the non-substituted primer. Thus, LNA when substituted at certain residues can allow the discrimination of highly homologous sequences.

**Index Terms**—Alternatively spliced isoforms, real-time PCR, locked nucleic acid (LNA)

## I. INTRODUCTION

ALTERNATIVE splicing is a widespread process used in higher eukaryotes to regulate gene expression and functional diversification of proteins. In a recent genome-wide microarray analyses, it is estimated that greater than 74% of the human multi-exon genes are alternatively spliced [1]. Compared with its wild-type counterpart, a splice variant can exhibit different enzymatic or signaling activities, novel subcellular localization and altered protein stability [2]. In order to understand the physiological and pathological contributions of alternative spliced isoforms, it is essential to determine the quantitative expression levels of each isoform.

Various methods have been used to quantify alternatively spliced isoforms, including Northern blotting, ribonuclease protection assay, semi-quantitative RT-PCR

and competitive RT-PCR. However, utilizations of these assays are restrained due to their intrinsic limitations of efficiency, sensitivity and specificity. Until recently, isoform-specific real-time PCR has been successfully applied for quantifying spliced variants with outstanding performance [3]-[6]. By designing primers or probes targeting the unique exon junctions of spliced isoforms, real-time PCR assay can be applied to discriminate these isoforms. Previously, we have successfully designed primers targeting exon junctions of mouse GFR $\alpha$ 2 isoforms to quantify the expression levels of these isoforms in mouse tissues [3]. However, if there is high sequence similarity between the unique exon junctions of the isoforms (e. g. human GFR $\alpha$ 2a and 2b), such design will suffer from significant cross amplification.

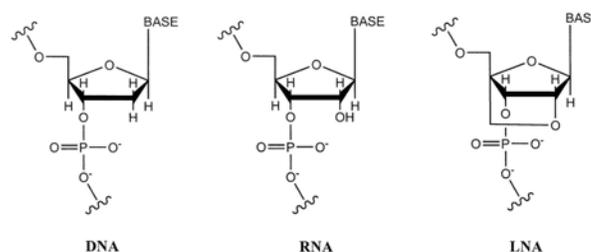


Fig. 1. Structural representations of DNA, RNA and LNA molecules.

In the present study, we developed an isoform-specific real-time PCR assay using exon-overlapping primers incorporated with locked nucleic acids (LNAs). LNAs are ribonucleotides containing a methylene bridge that connects the 2'-oxygen of ribose with the 4'-carbon (Fig. 1). This bridge results in a locked 3'-endo conformation that reduces the conformational flexibility of the ribose. The incorporation of LNA into a DNA primer increases the melting temperature ( $T_m$ ) and improves the hybridization affinity for complementary sequences [7]. Using human GFR $\alpha$ 2b isoform as a model, we demonstrate that LNA substituted primers allow the specific amplification of the desired sequences over highly homologous sequences by at least 100,000-fold.

The specificity of each LNA substituted primer is highly dependent on the position and the number of LNA substitutions. We found that two consecutive LNA substitutions 5' preceding the sequence difference have maximal effect on specificity of the primer.

## II. MATERIALS AND METHODS

### A. Templates and Primers

Open reading frame of human GFR $\alpha$ 2 isoforms were cloned into p-GEMT vector (Promega). Plasmids linearized with XmnI (Promega) were used as PCR templates. DNA primers were designed using DNAMAN software. Primers with specific LNA substitutions have identical sequence as the DNA counterparts. All the primers are listed in Table 1.

**Table 1.** List of primers used in this study.

Label	Sequence
P553R	gcagatggagatgtaggaggag
A+5-L0	tgcccttcttcttctagacgag
C+5-L0	tgcccttcttcttctagggaca
B+5-L0	tgcccttcttcttctaggtgag
B+5-L1	tgcccttcttcttctaggtgaG
B+5-L2	tgcccttcttcttctaggtgAg
B+5-L3	tgcccttcttcttctaggtGag
B+5-L4	tgcccttcttcttctaggtGgag
B+5-L5	tgcccttcttcttctaggtGtgag
B+5-L6	tgcccttcttcttctaggtGgtgag
B+5-L7	tgcccttcttcttctaggtGgtgag
B+5-L8	tgcccttcttcttctaggtGgtgag
B+5-L45	tgcccttcttcttctaggtGTgag
B+5-L67	tgcccttcttcttctaggtAGgtgag
B+5-L16	tgcccttcttcttctaggtGgtgaG
B+5-L123	tgcccttcttcttctaggtGAG

LNA substitutions are denoted as capital letters.

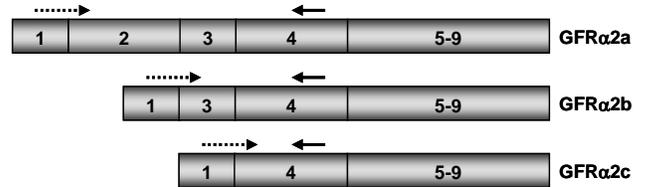
### B. Real-Time PCR

Sequence-independent real-time PCR using SYBR Green I was performed on iCycler iQ (Biorad, Hercules, USA). Fifty microliter PCR reaction for amplifying GFR $\alpha$ 2 isoforms was performed in 1X XtensaMix-SG<sup>TM</sup> (BioWORKS) containing 2.5 mM MgCl<sub>2</sub>, 100 nM of each primer and 0.5 U DyNAzyme II (Finnzymes, Finland). The PCR condition consists of initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 1 min, 60°C for 30 s and 72°C for 1 min. Real-time detections were carried out at annealing stages. Melting curves were generated after amplification. The threshold cycles (Ct) were calculated using the Optical interface iQ5 software.

## III. RESULTS

### A. Specificity of Exon Overlapping DNA Primers for GFR $\alpha$ 2 Isoforms

The human GFR $\alpha$ 2 gene is known to produce three known isoforms by alternative splicing of pre-mRNA. GFR $\alpha$ 2a consists of all the exons from 1 to 9, whereas GFR $\alpha$ 2b and GFR $\alpha$ 2c do not have exon 2 and exon 2-3, respectively (Fig. 2). Due to the absence of unique exons in GFR $\alpha$ 2b and GFR $\alpha$ 2c isoforms, specific detection of these transcripts can only be achieved using real-time PCR primers targeting the unique exon junctions.

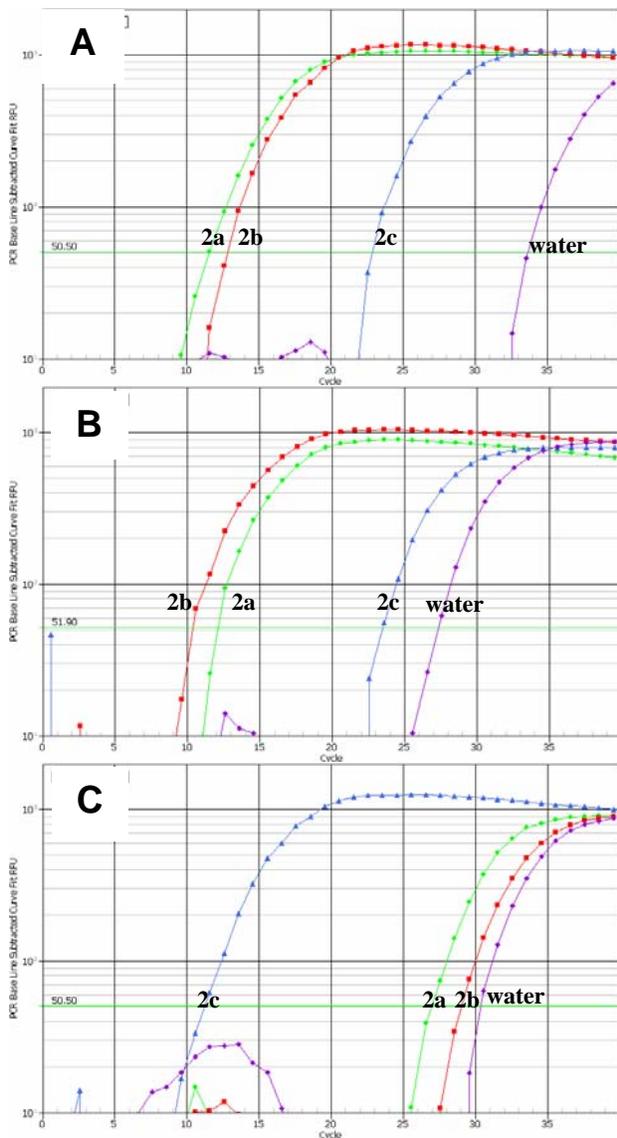


**Fig. 2.** Exon organizations of human GFR $\alpha$ 2 isoforms. Isoform-specific forward primers were designed to overlap the unique exon junction of each isoform (dashed arrows). A common reverse primer was used to amplify all the isoforms (solid arrow).

Firstly, we adopted the previous design strategy for mouse GFR $\alpha$ 2 isoforms, using primers with 5 base pairs (bp) crossing the unique exon junction of each isoform [3]. The Ct values for specific and non-specific amplification of the primers were listed in Table 2. Although this strategy was successfully applied to discriminate GFR $\alpha$ 2c from GFR $\alpha$ 2a and GFR $\alpha$ 2b by 100,000-fold (Table 2 and Fig. 3C), discrimination between GFR $\alpha$ 2a and GFR $\alpha$ 2b was extremely poor (Table 2). Specific DNA primer for GFR $\alpha$ 2b (B+5-L0) cross amplified GFR $\alpha$ 2a isoform with discrimination level lower than 5-fold (Fig. 3B). Likewise, A+5-L0 also mis-amplified GFR $\alpha$ 2b isoform significantly (Fig 3A). Therefore, the design strategy of previous study could not be successfully applied to discriminate human GFR $\alpha$ 2a and GFR $\alpha$ 2b isoforms.

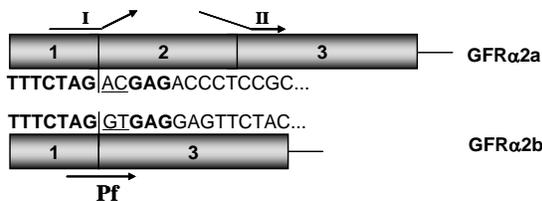
**Table 2.** specific and non-specific amplification of exon overlapping primers for GFR $\alpha$ 2 isoforms

Primers	Threshold Cycle (Ct)			
	2a	2b	2c	water
<b>A+5-L0</b>	<b>11.6</b>	12.8	22.8	33.6
<b>B+5-L0</b>	12.0	<b>10.2</b>	23.4	27.3
<b>C+5-L0</b>	26.9	29.0	<b>11.2</b>	30.3



**Fig. 3.** Amplification of GFR $\alpha$ 2 isoforms using primers A+5-L0 (A), B+5-L0 (B) and C+5-L0 (C).

Sequence alignment of GFR $\alpha$ 2a and GFR $\alpha$ 2b isoforms reveal exon junctions 1/2 and 1/3 to have significant sequence similarity (Fig. 4). As a result, primer B+5-L0 can mis-prime to GFR $\alpha$ 2a template at position I, further extension of this primer towards exon 3 may render it to mis-prime at position II (Fig. 4).



**Fig. 4.** Challenge of designing specific primers for GFR $\alpha$ 2b isoform.

Because GFR $\alpha$ 2a possess the unique exon 2, specific primer can be designed within this exon. Conversely, the design for GFR $\alpha$ 2b is limited to exon junction 1/3. In order to specifically quantify GFR $\alpha$ 2b isoform with

constrained primer design, modifications of this DNA primer are required to maximize its specificity.

### B. Improved Specificity of the Primers with LNA Substitutions

Previous studies have shown that LNA substitutions can increase the hybridization temperature and increase the specificity to complementary template [8], [9]. Therefore, we designed a set of primers for GFR $\alpha$ 2b with identical sequence (B+5) but different LNA substitutions (Table 1). Briefly, single LNA was placed at first to eighth residue from 3' of the primer. Sequence differences between GFR $\alpha$ 2a and GFR $\alpha$ 2b resides at fourth and fifth residue. Primers with multiple LNA substitutions were also synthesized. The specificity of these primers is measured together with the DNA primer control (Table 3). In contrast to the other reports [8], [10], 3' terminal LNA substitution (L1) did not affect the PCR efficiency as seen with similar Ct values between DNA and LNA primers (2b). However, PCR was inhibited using primers L16 and L123. About 100-fold increase in specificity was achieved by single LNA substitution at first (L1) and sixth (L6) residue. LNA substitutions at the fourth and fifth residues (L45) did not improve the specificity significantly. Consecutive LNA substitutions at sixth and seventh residues (L67) dramatically improved the discrimination by 19 cycles, which corresponds to 100,000-fold (Fig. 5).

**Table 3.** Specificity of B+5 primers with LNA substitutions

Primer	Ct (2b)	Ct (2a)	$\Delta$ Ct
L0	10.2	12.0	1.8
L1	11.7	18.5	6.9
L2	11.4	14.9	3.5
L3	11.7	14.5	2.8
L4	11.4	13.5	2.1
L5	11.4	12.3	0.9
L6	12.4	18.9	6.5
L7	11.7	16.4	4.7
L8	11.4	13.6	2.2
L45	12.4	17.2	4.8
L67	12.5	31.2	18.7
L16	*	*	*
L123	*	*	*

\* No amplification was observed using primers with L16 and L123 LNA substitutions.

In conclusion, despite the extremely poor specificity of B+5 DNA primer, LNA substitutions in this primer can improve its specificity dramatically. Discrimination of highly homologous alternative spliced isoforms can be achieved by appropriate substitutions of LNA residues in the exon overlapping primers.

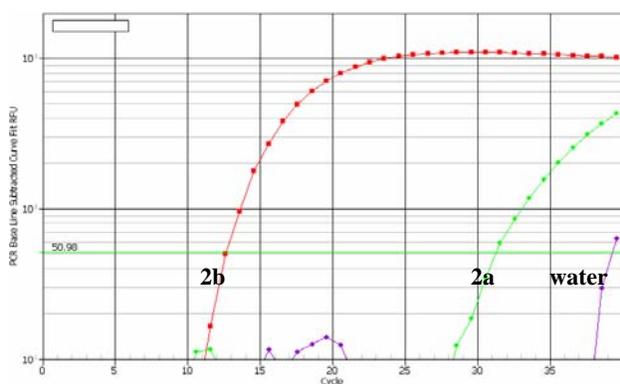


Fig. 5. Specific amplification of GFR $\alpha$ 2b using primer B+5-L67.

#### IV. DISCUSSION

Alternative splicing event is believed to contribute to the expression and functional diversification of a limited set of genes of a genome. In order to understand the physiological contributions of the isoforms, quantitative expression of these isoforms becomes essential. Because of the mechanism of splicing, shorter isoforms usually lack unique exons as targets for specific quantification. Sequence similarity at unique exon junctions poses more difficulty in primer design for real-time PCR quantification.

In this study, we presented the challenge in designing specific primers for alternatively spliced isoforms and demonstrated the application of LNA substituted primers to achieve desired assay specificity. Compared to the DNA primers which showed extremely poor discrimination of homologous isoforms, single LNA substitution can improve the specificity depending on the position of substitution. Although 3' terminal LNA substitution has been shown to severely inhibit PCR, we did not observe such effect. Conversely, terminal LNA substitution has significantly improved the discrimination. Such contradiction could be due to sequence variation at 3' terminal or differences in the DNA polymerases used as discussed previously [8]. Multiple LNA substitutions led to identification of highly specific LNA/DNA chimeric primer that could achieve 100,000-fold specificity against highly homologous template. It is interesting that this primer contains two consecutive LNA residues 3' preceding the sequence differences of the isoforms. However, substitution of the residues with sequence differences with LNAs did not improve specificity significantly. It has been observed that the DNA sugar 3' of the LNA is driven to a C3'-endo conformation as shown by NMR structures of DNA/LNA chimeric oligos complementary to RNA [11]. Therefore, the substituted LNA residues may induce conformational changes in the adjacent DNA ribose moiety, thus improving the affinity of these DNA residues to perfect match sequences. The predictive rules governing LNA substituted primers for high specificity amplification of DNA template remains to be elusive.

LNA/DNA chimeric probes designed for SNP genotyping analysis has been widely reported [12], [13]. This is the first report demonstrating the application of LNA/DNA chimeric primers in the discrimination of alternatively spliced isoforms. In summary, LNA substituted primers can exhibit outstanding specificity compared to their DNA primer counterparts. We have identified a highly specific LNA substituted primer for GFR $\alpha$ 2b isoform. We suggest that LNA molecules could be applied similarly to other highly homologous alternative spliced isoforms where limited DNA primer designs are available.

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