



<Research Article>

Detection of short DNA using ligase chain reaction with LNA-modified probes, and its application to the phosphorothioate oligonucleotide fomivirsen

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Summary In this study we developed a method for detecting short DNA using ligase chain reaction (LCR) with locked nucleic acid (LNA)-modified probes. To optimize LCR for short DNA detection, the effect of the probe length was examined using probe sets composed of 10-, 11-, 12-, 14-, and 20-bases of deoxyoligonucleotides. LCR using probe sets of 11 bases or longer (excluding the 10-base set) successfully detected target DNA sequences consisting of 22 or more bases. To examine the effect of LNA modification on LCR, LNA was inserted at the 8th, 11th, or 14th positions from the ligation site of each 14-base deoxyoligonucleotide probe. LNA modification at the 8th position suppressed amplification, whereas modification at the 11th position had no inhibitory effect, and that at the 14th position facilitated it. Furthermore, when LNA was inserted at the terminal opposite to the ligation site in probe sets of 10 to 14 bases, the LNA-modified probe sets (except for the 10-base set) facilitated amplification compared with the unmodified ones. These results indicate that LCR can detect short DNA consisting of 22 or more bases, and that the use of probes modified with LNA far from the ligation site facilitated amplification. Finally, this LCR method was applied to detect fomivirsen, a 21-phosphorothioate deoxyoligonucleotide, used as an oligonucleotide therapeutic, and successfully detected it at a concentration of 6.3 nmol/L. Accordingly, this LCR method is also useful for detecting phosphorothioate deoxyoligonucleotides.

Key words: Fomivirsen, Ligase chain reaction, Locked nucleic acid, Oligonucleotide therapeutic, Phosphorothioate oligonucleotide

1. Introduction

Currently, short oligonucleotides, including functional nucleic acids such as antisense oligonucleotides (ASOs), microRNAs (miRNAs), and aptamers, have gained significant attention as next-generation therapeutic and diagnostic agents¹. In

particular, the clinical development of ASOs has rapidly expanded, and simple and sensitive methods to monitor their pharmacokinetics and stability in biological fluids have been required. Moreover, the detection of degraded DNA from forensic or clinical samples has also represented a similar analytical challenge^{2,3}.

The polymerase chain reaction (PCR) method

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has been widely utilized for the amplification and detection of gene sequences. However, conventional PCR has limitations when applied to short DNA targets. Because the primers are typically around 20 bases in length, binding two primers to a short target sequence of 20 to 30 bases causes severe steric hindrance or insufficient hybridization length. Consequently, extension reactions cannot occur efficiently, and then standard PCR amplification is not possible for short oligonucleotides. Although modified PCR methods, such as stem-loop RT-PCR, have been developed for small RNAs⁴, they often require complex primer designs and multiple enzymatic steps, which can increase costs. Alternative non-enzymatic methods, such as high-performance liquid chromatography (HPLC) and mass spectrometry (MS), have high accuracy but suffer from low throughput and high operational costs⁵.

We have previously investigated detection methods for short oligonucleotides that combine nuclease or ligase reactions with PCR^{6,7}. However, these methods required a two-step reaction, an initial enzymatic reaction (nuclease digestion or ligation) followed by transfer or reagent addition for subsequent PCR amplification, which increased hands-on time and the risk of carryover contamination. To overcome these limitations, we decided to focus on ligase chain reaction (LCR). In LCR, double-stranded DNA was heat-denatured into single strands, allowing four discrete probes (5' and 3' forward probes, and 5' and 3' reverse probes) to specifically anneal to adjacent positions on the target sequence⁸⁻¹⁰. A thermostable DNA ligase then covalently joined the adjacent probes, forming a continuous single strand that serves as a template in subsequent cycles. Unlike PCR, LCR relies strictly on the direct annealing and ligation of probes across the target sequence, eliminating the need for a template extension step. Therefore, LCR possesses a theoretically superior suitability for the detection of short oligonucleotides.

Nevertheless, adapting LCR for short target detection introduced a critical trade-off. Reducing the length of the four individual probes to fit within a 20-to-30-base target lowered their melting

temperatures (T_m). This decrease destabilized probe-target hybridization at standard ligation temperatures, leading to a significant reduction in amplification efficiency. Therefore, we focused on the application of locked nucleic acid (LNA). LNA is an RNA analog in which the furanose ring of the ribose sugar is chemically locked in an RNA-mimicking conformation by the introduction of an $O2',C4'$ -methylene linkage¹¹ (Fig. 1). The incorporation of LNA into an oligonucleotide drastically increased the T_m and binding affinity toward complementary strands. This stabilization is attributed to the pre-organized C3'-endo conformation, which restricts the conformational flexibility of the nucleic acid backbone¹¹.

In this context, short oligonucleotides are widely utilized as functional nucleic acids, particularly in oligonucleotide therapeutics. To ensure the safe and effective use of these therapeutics, bioanalysis is essential, and LCR holds potential as a foundational technology for this purpose. Therefore, fomivirsen, the world's first FDA-approved antisense medicine, was selected as a standard model target for phosphorothioate (PS)-modified ASOs, making it an ideal compound to demonstrate the utility of our detection method for oligonucleotide therapeutics.

In this study, we performed a basic study of an LCR method for detecting short DNA sequences using DNA probes designed shorter than the length of 20 to 30 bases used in general LCR methods⁸⁻¹⁰. We investigated the length of the probes and the introduction sites of LNA, and applied LCR to the

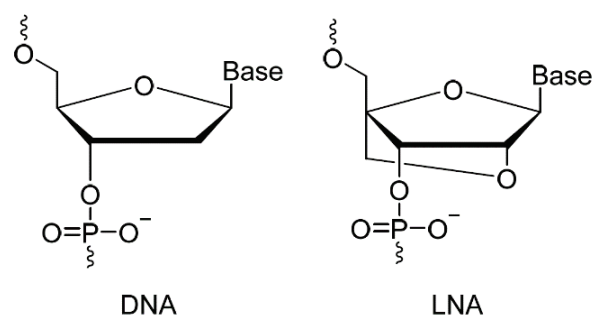


Fig. 1. Chemical structure of DNA and LNA.

Table 1. Sequences of the oligonucleotides and probes used in this study.

Length	Forward probes (F/FP) (5' → 3')	Reverse probes (R/RP) (5' → 3')
Unmodified (λ DNA)		
10-base	F1-10: ggctcatgct	R1-10: tcagactgtg
	FP1-10: P-cacagtctga	RP1-10: P-agcatgagcc
11-base	F1-11: gggctcatgct	R1-11: ctgagactgtg
	FP1-11: P-cacagtctgag	RP1-11: P-agcatgagccc
12-base	F1-12: ggggctcatgct	R1-12: gctcagactgtg
	FP1-12: P-cacagtctgagc	RP1-12: P-agcatgagcccc
14-base	F1-14: cgggggctcatgct	R1-14: ccgctcagactgtg
	FP1-14: P-cacagtctgagcgg	RP1-14: P-agcatgagcccccg
20-base	F1-20: ataacacgggggctcatgct	R1-20: gttgaaccgctcagactgtg
	FP1-20: P-cacagtctgagcgggtcaac	RP1-20: P-agcatgagcccccggttat
LNA internal (14-base)		
L8 position	F2-14-L8: cgggggCtcatgct	RP2-14-L8: P-agcatgaGcccccg
L11 position	F2-14-L11: cggGggctcatgct	RP2-14-L11: P-agcatgagccCccg
L14 position	F2-14-L14: Cgggggctcatgct	RP2-14-L14: P-agcatgagccccG
LNA terminal		
10-base	F3-10-L: Ggctcatgct	R3-10-L: Tcagactgtg
	FP3-10-L: P-cacagtctgA	RP3-10-L: P-agcatgagcC
11-base	F3-11-L: Gggctcatgct	R3-11-L: Ctcagactgtg
	FP3-11-L: P-cacagtctgaG	RP3-11-L: P-agcatgagccC
12-base	F3-12-L: Ggggctcatgct	R3-12-L: Gctcagactgtg
	FP3-12-L: P-cacagtctgagC	RP3-12-L: P-agcatgagcccC
14-base	F3-14-L: Cgggggctcatgct	R3-14-L: Ccgctcagactgtg
	FP3-14-L: P-cacagtctgagcgG	RP3-14-L: P-agcatgagccccG
Fomivirsén		
Fomivirsén	gcgtttgctcttcttcttgcg	
Probes	F4: Gcgtttgctc	R4: Cgcaagaagaa
	FP4: P-ttcttcttgcG	RP4: P-gagcaaacgC

Uppercase letters indicate LNA modifications. "P-" indicates phosphorylation at the 5' end. Sequences are shown from 5' to 3'. For the LNA internal probes, unmodified 14-base FP and R probes were used. Fomivirsén consists of 21 phosphorothioate linkages.

detection of fomivirsén, an oligonucleotide therapeutic which has 21-base phosphorothioate-modified DNA.

2. Materials and Methods

All oligonucleotides were synthesized and modified (uppercase letter and P in the sequence indicated LNA modification and phosphorylation at

the 5' ends) by Gene Design (Osaka, Japan). These sequences are listed in Table 1. Probe sets composed of 10-, 11-, 12-, 14-, and 20-base deoxyoligonucleotides were designed based on a λ DNA sequence (GI: 9626243, positions 21561–21600) (Fig. 2A). This specific region was selected as a standard model template because it has a balanced GC content (55%) and lacks repetitive sequences, making it suitable for evaluating probe design

parameters in LCR. LNA was inserted at the 8th, 11th, and 14th positions from the ligation site on probes F1-14 and RP1-14 (Fig. 2B). Next, 10-, 11-, 12-, and 14-base deoxyoligonucleotides, modified with LNA at the terminal opposite to the ligation site, were used (Fig. 2C). The 21-phosphorothioate deoxyoligonucleotide fomivirsen (ISIS 2922) was synthesized with the following sequence: 5'-gcgtttgctcttcttcttcgcg-3'¹². The probe set for the detection of fomivirsen was also designed (Fig. 2D).

In LCR for λ DNA sequence, a reaction mixture (10 μ L) contained 16 U of Taq DNA ligase

(New England Biolabs, MA, USA), 1 $\times$ ligase buffer, 0.3 μ mol/L of each probe, 1 $\times$ SYBR Green I Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, USA), and 1 ng of λ DNA (Nippon Gene, Tokyo, Japan). LCR was performed using a StepOnePlus Real-time PCR System (Thermo Fisher Scientific, MA, USA) with the following thermal cycle program: 120 s at 95°C and 60 cycles of amplification each consisting of 20 s at 95°C, 40 s at 56°C, and 20 s at 70°C. In the LCR for fomivirsen, the reaction mixture (10 μ L) contained 16 U of Taq DNA ligase, 1 $\times$ ligase buffer, 0.5 μ mol/L of each

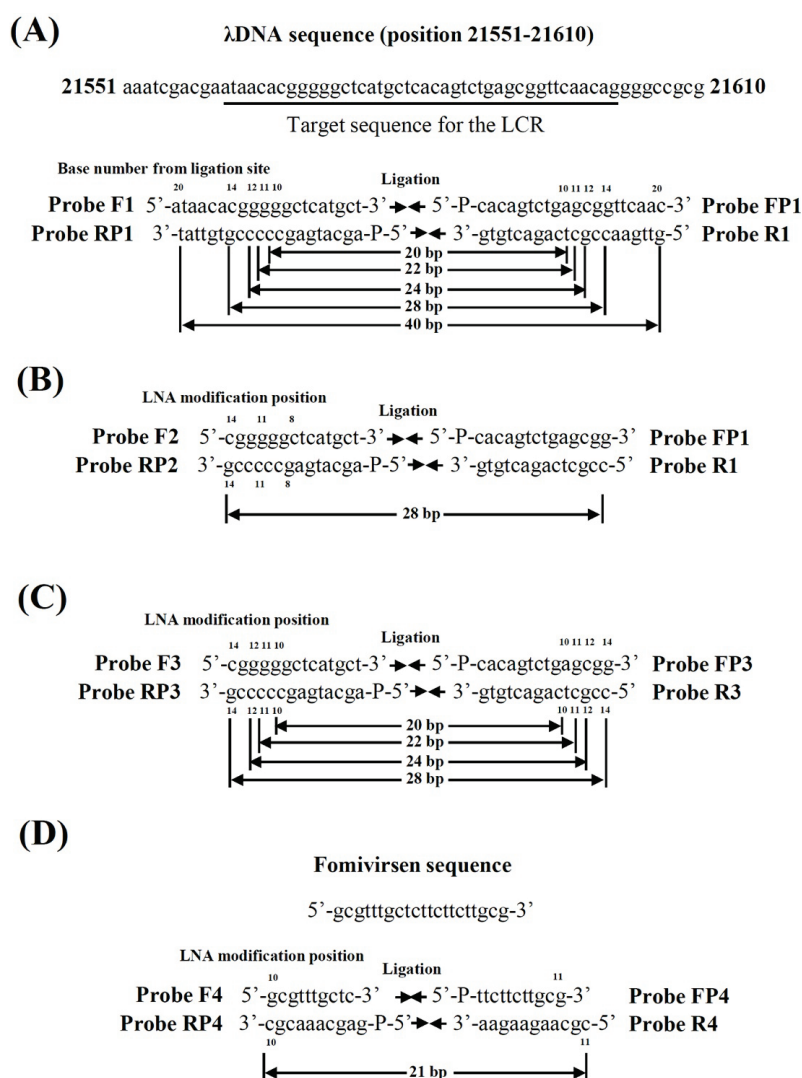


Fig. 2. Probes used in LCR. (A) Probes for the model λ DNA sequence. Probe sets comprising F1, FP1, R1, and RP1 were synthesized as 10-, 11-, 12-, 14-, and 20-base deoxyoligonucleotides targeting the λ DNA sequence. (B) 14-base probes (F2 and RP2) with LNA inserted at the 8th, 11th, and 14th positions from the ligation site. (C) Probe sets (F3, FP3, R3, and RP3; 10, 11, 12, and 14 bases) modified with LNA away from the ligation site. (D) Probes modified with LNA away from the ligation site for fomivirsen.

probe, 1×SYBR Green I Nucleic Acid Gel Stain, and 1 µL of fomivirsen sample (0, 1.6, 6.3, 25, and 100 nmol/L of water solution). LCR was performed using a StepOnePlus Real-time PCR System with the following thermal cycle program: 120 s at 95°C and 60 cycles of amplification each consisting of 20 s at 95°C, 40 s at 56°C, and 20 s at 68°C. In the optimization of reaction conditions for fomivirsen, a temperature of 68°C was adopted because it suppressed non-specific amplification while achieving high amplification efficiency.

After LCR amplification, 10 µL of the LCR sample was electrophoresed on 15% polyacrylamide gels with 0.025 mol/L Tris–0.19 mol/L glycine buffer (pH 8.3). Following electrophoresis, the gel was imaged using Gel Doc XR+ with Image Lab Software (Bio-Rad, CA, USA). A 10 bp DNA step ladder (10–100 bp) (Fujifilm Wako, Osaka, Japan) was utilized as a size marker.

3. Results and discussion

To examine the effects of DNA probe length and LNA modification on amplification in LCR, λ DNA was used as a model DNA. The probes used for LCR in this study are illustrated in Fig. 2. Using these probes, we attempted to detect short sequences ranging from 20 to 40 bases.

Polyacrylamide gel electrophoresis (PAGE) analysis after LCR revealed that no product bands were formed with the 10-base DNA probe set. In contrast, a 22-base product band was faintly detected with the 11-base probe set, while 24-, 28-, and 40-base products were clearly observed with the 12-, 14-, and 20-base probe sets, respectively (Fig. 3). These results suggest that a minimum probe length of 11 bases was required for LCR-based detection using DNA probes. Although remarkable amplification was observed with the 20-base probe, the primary objective of this study was to detect shorter target sequences. Therefore, we next examined the effects of LNA modification on amplification using probes of 14 bases or shorter.

First, the effect of the LNA modification site was evaluated using 14-base probes. When an LNA

modification was introduced into one of the four LCR probes at positions 8, 11, or 14 bases away from the ligation site, amplification was suppressed with the probe modified at the 8-base position, whereas it was facilitated at the 14-base position (Fig. 4). This indicates that LNA modification near the ligation site inhibited the ligation reaction. As documented for other ligases, the T4 DNA ligase has been reported to contact seven to nine bases on the 5'-phosphate terminus (donor side) of the nick and three to five bases on the 3'-hydroxyl group (acceptor side)¹³. In contrast, when located 14 bases away from the ligation site, the LNA modification was considered to facilitate LCR amplification by enhancing hybridization binding affinity.

Next, we investigated using 10-, 11-, 12-, and 14-base probes modified with LNA at the terminal opposite to the ligation site. No bands were observed with the 10-base probe, whereas product bands of 22, 24, and 28 bases were detected with the 11-, 12-, and 14-base probes, respectively (Fig. 5). Similar to the unmodified DNA probes, the lack of amplification with the 10-base LNA-modified probe indicated that LNA incorporation had no effect on the minimum probe length required for LCR. On the other hand, the LNA-modified 11-, 12-, and 14-base probes, particularly the 14-base probe, showed enhanced amplification when compared with the corresponding DNA probes in Fig. 3. This demonstrated that LNA modification at the terminal opposite to the ligation site was effective for the LCR-based detection of short sequences.

Finally, based on these findings, we attempted to detect fomivirsen, an ASO consisting of a 21-base phosphorothioate-modified DNA, using LCR. When LCR was performed using two 10-base probes and two 11-base probes, each modified with LNA at the terminal opposite to the ligation site, a 21-base product was observed as bands that increased in intensity in a concentration-dependent manner at fomivirsen concentrations of 6.3, 25, and 100 nmol/L, respectively (Fig. 6). In Figs. 3 and 5, no products were detected with the 10-base probe set (20-base target), whereas amplification was achieved with the 11-base probe set (22-base target), indicating that

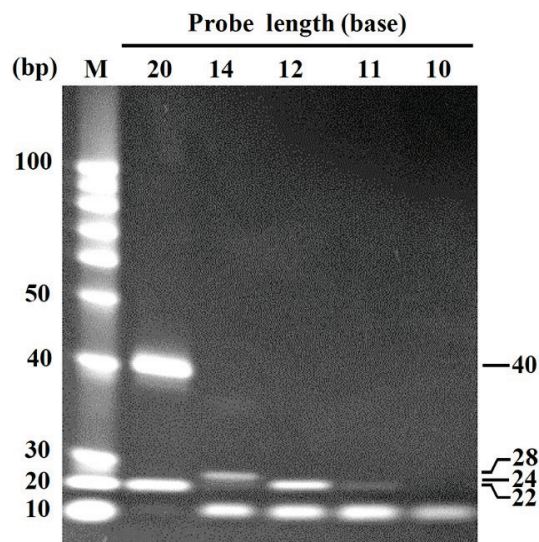


Fig. 3. PAGE analysis of LCR products for the λ DNA target using 10-, 11-, 12-, 14-, and 20-base probe sets. The predicted sizes of the amplification products were 20, 22, 24, 28, and 40 bp, respectively. The minor band observed around 10 bp is presumed to correspond to unreacted LCR probes. Lane M, DNA size marker.

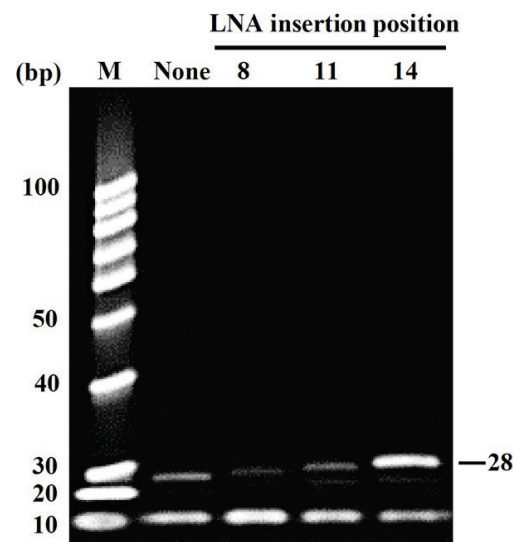


Fig. 4. PAGE analysis of LCR products for the λ DNA target using 14-base probes with LNA insertions at the 8th, 11th, and 14th positions from the ligation site. The predicted product size was 28 bp. LCR products obtained using the 14-base probes without LNA are shown as None. The minor band observed around 10 bp is presumed to correspond to unreacted LCR probes. Lane M, DNA size marker.

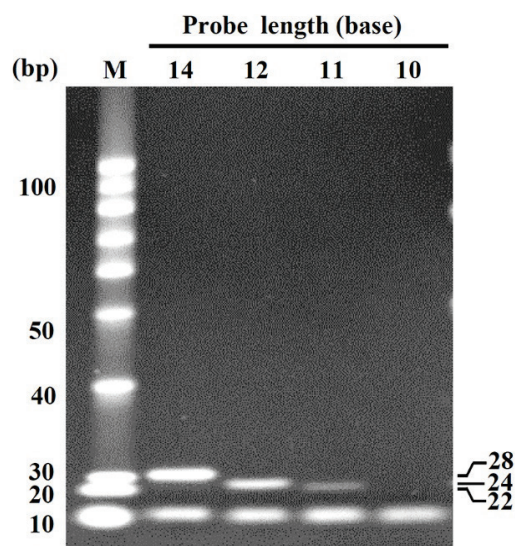


Fig. 5. PAGE analysis of LCR products for the λ DNA target using 10-, 11-, 12-, and 14-base probes modified with LNA at the terminal opposite to the ligation site. The predicted product sizes were 20, 22, 24, and 28 bp, respectively. The minor band observed around 10 bp is presumed to correspond to unreacted LCR probes. Lane M, DNA size marker.

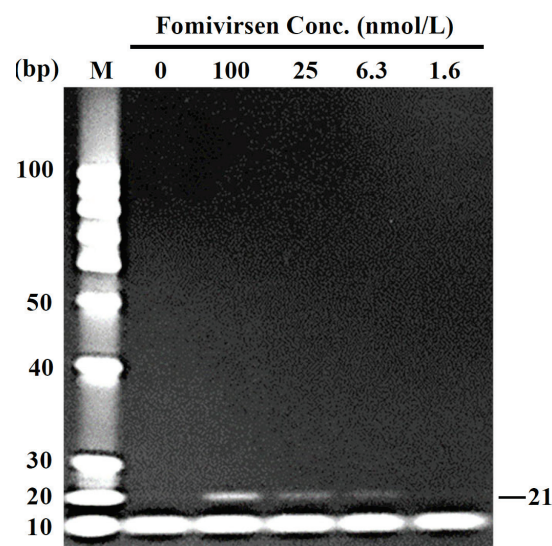


Fig. 6. PAGE analysis of LCR products for fomivirsen targets using probes modified with LNA at the terminal opposite to the ligation site. LCR products obtained at fomivirsen concentrations of 0, 1.6, 6.3, 25, and 100 nmol/L are shown. The predicted product size was 21 bp. The minor band observed around 10 bp is presumed to correspond to unreacted LCR probes. Lane M, DNA size marker.

ligation does not proceed efficiently with 10-base probes alone. In contrast, 21-base fomivirsen was successfully detected using a probe set comprising two 10-base probes and two 11-base probes. In this reaction, fomivirsen acts as a phosphorothioate-modified DNA splint (template) that aligns the adjacent probes for ligation. This result was consistent with a previous report demonstrating that Taq DNA ligase can ligate DNA probes with a phosphorothioate-modified DNA splint⁷. Consequently, this study demonstrated that short phosphorothioate-modified DNA could be detected by LCR using Taq DNA ligase. On the other hand, the current protocol requires 60 cycles, where potential non-specific ligation challenges real-time quantitative LCR. Future work will focus on optimizing ligases, buffers, and LNA positions to enhance amplification efficiency and enable real-time quantitative LCR, followed by a comprehensive validation including the assessment of accuracy, precision, specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, and range.

4. Conclusion

When applying LCR to detect short DNA, such as ASO, shortening the DNA probes decreased their T_m values, which consequently reduced amplification. Therefore, the incorporation of LNAs was effective. Because LNA introduction near the ligation site inhibited the ligation reaction, modifying the terminal opposite to the ligation site was considered optimal. Furthermore, although LCR using LNA-modified probes could not detect a 20-base sequence, it successfully detected fomivirsen, which was composed of 21-base phosphorothioate-modified DNA. Overall, this study provides valuable insights for the detection of short DNA using LCR.

Conflicts of interest

The authors declare no conflict of interest.

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