J|A|C|S COMMUNICATIONS

Published on Web 06/06/2008

# Polymerase Chain Reaction and Transcription Using Locked Nucleic Acid Nucleotide Triphosphates

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Locked nucleic acid (LNA) has demonstrated unique applicability within numerous areas of chemical biology,[1,2] but enzymatic synthesis by DNA and RNA polymerases has been largely unexplored. As a first step in this direction we recently reported preliminary results of primer extension reactions using LNA nucleotide triphosphates.[3,4] As different DNA and RNA polymerases vary in their discrimination of modified nucleotide triphosphates we examined many polymerases and found that Phusion High Fidelity DNA Polymerase was shown to readily accept LNA-TTP and LNA-ATP. We herein apply LNA nucleotide triphosphates to introduce polymerase chain reaction (PCR) amplification of LNA-containing DNA using 9°$N_m$ DNA polymerase and transcription using T7 RNA polymerase.

First, we examined the incorporation of LNA-T and LNA-A nucleotides (Figure 1, B = thymin-1-yl and adenine-9-yl) into a DNA strand from a DNA template using 9°$N_m$ DNA polymerase. The template directs six incorporations of LNA-T and LNA-A nucleotides, three of each, mixed with incorporations of DNA-C and DNA-G nucleotides (Figure 2A). A positive control reaction (using all four natural dNTPs) and negative control reaction (with a mixture lacking the nucleotide triphophates similar to the LNA nucleotide triphophates that were to be tested for incorporation) were performed in parallel to the reactions involving the incorporations of LNA monomers. The experiment showed that 9°$N_m$ DNA polymerase accepts LNA nucleoside 5′-triphosphates as substrates (Figure 2C), like we have previously reported for Phusion High Fidelity DNA polymerase.[3] The products were further characterized by MALDI-TOF MS analysis to verify fidelity of LNA incorporations (Figure S2, Supporting Information). Notably, product degradation was observed after 30 min of reaction in the control reaction using all natural dNTPs (Figure S3). Such degradation was not observed in the reactions using LNA nucleotides, which we explain as nucleolytic stability induced by the LNA monomers.

As the next step we investigated incorporation of LNA-T and LNA-A nucleotides using an LNA-modified DNA template with LNA-nucleotides positioned opposite to the enforced LNA nucleotide incorporation sites. Furthermore, the incorporation of DNA nucleotides opposite to the LNA nucleotides of the template strand was investigated (T4, Table S1; incorporation of three LNA-T followed by three LNA-A nucleotides mixed with DNA-C and DNA-G nucleotides). This experiment demonstrated that both Phusion High Fidelity DNA polymerase and 9°$N_m$ DNA polymerase are capable of incorporating LNA and DNA nucleotides directly opposite to both an LNA or a DNA nucleotide in a template strand and to further extend the strand to full-length (Figure S4).

Encouraged by these results, we investigated the amplification of LNA-modified DNA sequences by PCR using Phusion High Fidelity DNA polymerase and 9°$N_m$ DNA polymerase. Template T1 and primers P2 and P3 (Figure 2B; see Supporting Information for reaction conditions) were designed to enforce incorporation of

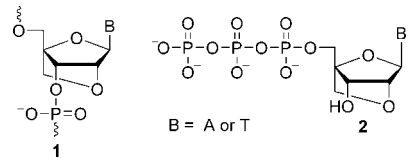


***Figure 1.*** Structural representation of an LNA monomer **1** and LNA nucleoside 5′-triphosphates **2**.

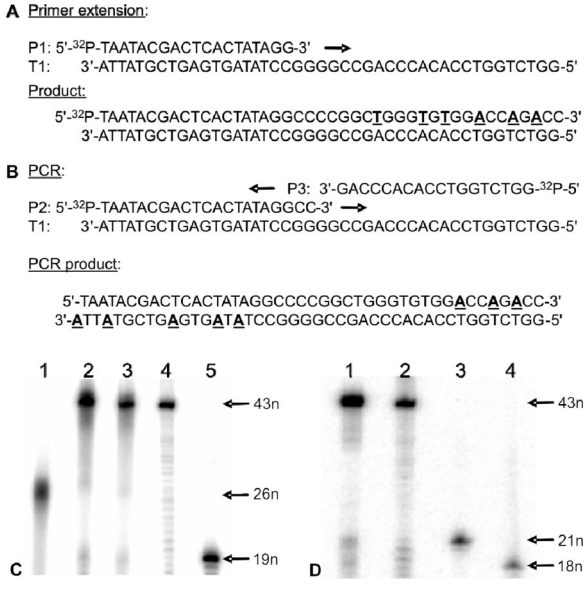


***Figure 2.*** (A and C) Enzymatic incorporation of LNA-T and LNA-A nucleotides using 9°$N_m$ DNA polymerase: P1, primer (19n); T1, template (43n). (Lane 1) Negative control reaction with only two natural nucleotides (dCTP and dGTP) present (26n); (lane 2) incorporation of LNA-T and LNA-A nucleotides (43n); (lane 3) positive control using all four natural deoxynucleotides (43n); (lane 4) 43n marker DNA; (lane 5) primer (19n). (B and D) PCR experiments with LNA-A nucleoside 5′-triphosphate: P2, forward primer (21n); P3, reverse primer (18n); T1, template (43n). (Lane 1) dATP was replaced with LNA-ATP; (lane 2) 43n marker DNA strand; (lane 3) forward primer (21n); (lane 4) reverse primer (18n). LNA incorporations are marked in bold and underlined in all product sequences.

LNA-A nucleotides toward the 3′-ends of the PCR product. The primers were 5′-$^{32}$P-labeled and purified by gel electrophoresis, and LNA-A nucleoside 5′-triphosphate was used as the LNA building block.

Amplified LNA-containing PCR product was observed in good yield using 9°$N_m$ DNA polymerase (Figure 2D), whereas Phusion High Fidelity DNA polymerase afforded the desired product in much lower yield (Figure S5A). The products were verified to be the expected full-length products by MALDI-TOF MS analysis (Figure S6). Notably, the corresponding PCR using all four dNTPs


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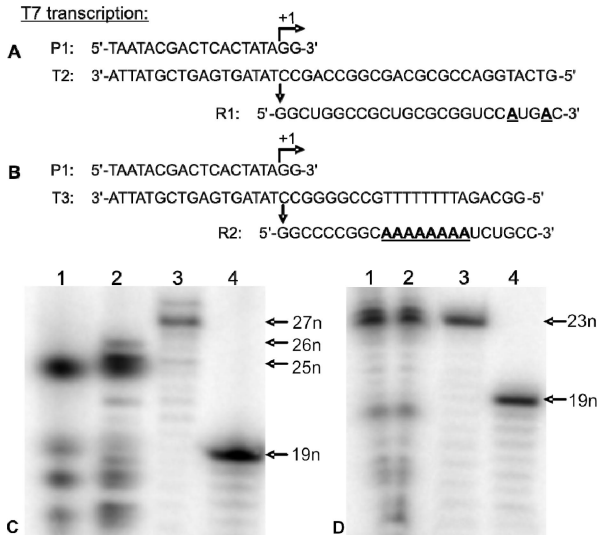


***Figure 3.*** Transcription studies using LNA-ATP and T7 RNA polymerase: (A and C) P1, T7 promoter (19n); T2, DNA template (42n); R1, RNA transcript (25n). (Lane 1) Reaction using LNA-ATP instead of rATP; (lane 2) reactions using all four natural rNTPs; (lane 3) 27n marker RNA strand; (lane 4) 19n T7 promoter. (B and D) P1, T7 promoter (19n); T3, DNA template (42n); R2, RNA transcript (23n). (Lane 1) reaction using LNA-ATP instead of rATP; (lane 2) reaction using all four natural rNTPs; (lane 3) 23n marker RNA strand; (lane 4) 19n T7 promoter. LNA incorporations are marked in bold and underlined in all product sequences.

and 9°$N_m$ DNA polymerase was unsuccessful (Figure S5B). We speculate again that the nucleolytic activity of the polymerase leads to strand degradation under the applied conditions unless LNA nucleotides are incorporated. The developed PCR thus allows selective amplification of LNA-containing sequences.

To further explore the applications of LNA nucleoside 5′-triphosphates as substrates for polymerases we investigated transcription with T7 RNA polymerase.[5] Transcription experiments[6] were carried out using the appropriate T7 promoter (P1, Figure 3A) and template (T2, Figure 3A) sequences to study the incorporation of LNA nucleotides in RNA strands. The first experiment showed that T7 RNA polymerase efficiently transcribed the template to the expected full-length LNA-containing RNA transcript (25n) having LNA incorporations at positions 21 and 24 (Figure 3C, lane 1). The corresponding control reaction with rATP (Figure 3C, lane 2) showed a minor 25n + 1n product as commonly reported for transcription reactions performed with T7 RNA polymerase.[7]

Multiple and successive incorporations of LNA-A nucleotides were likewise investigated using T7 RNA polymerase. For this experiment, we employed a template DNA strand with a segment encoding for continuous incorporation of eight LNA-A nucleotides (T3, Figure 3B). Remarkably, transcription proceeded efficiently to afford the full-length transcript (R2, 23n; Figure 3D).

To use LNA in RNA selection studies utilizing PCR, the RNA polymerase must be able to read an LNA-modified DNA template. This was investigated using template T4 (Table S1). This template was designed to force incorporation of three LNA-A nucleotides, with the first LNA-A in positions complementary to a natural RNA nucleotide and the next ones opposite to LNA nucleotides. Moreover, the template was designed to enforce two incorporations of natural RNA nucleotides in positions complementary to LNA-modified nucleotides in the template. The results showed a full-length transcript (R3, 26n, Table S1; Figure S7), and thus clearly demonstrated the versatility of T7 RNA polymerase mediated transcription involving LNA nucleotides as substrates and as template strand constituents.

PCR amplification and transcription of LNA-type oligonucleotides using DNA and RNA polymerases and LNA nucleotide triphosphates have been realized. These developments promise future exploration of the conformationally locked LNA nucleotides as constituents in large RNA molecules and as building blocks for the generation of aptamers[8] via SELEX.[9]

**Acknowledgment.** We thank Lykke H. Hansen and Associate Professor Finn Kirpekar for their technical assistance with MALDI-TOF MS analysis. The Nucleic Acid Center is a research center of excellence funded by the Danish National Research Foundation for studies on nucleic acid chemical biology. We thank the Danish National Research Foundation for financial support.

**Supporting Information Available:** Gel analysis of LNA incorporation, MALDI-TOF MS spectra, and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA801389N