

De Novo Synthesis of PCR Templates for the Development of SARS Diagnostic Assay

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Abstract

A novel coronavirus was identified as the cause for severe acute respiratory syndrome (SARS). The complete sequence of SARS genome has provided an opportunity for the development of molecular diagnostic assays. To restrain further outbreak of SARS, the World Health Organization has posted several pairs of polymerase chain reaction (PCR) primers for early diagnosis and urged more research to be done on PCR protocols. Here we report a strategy for the *de novo* synthesis of PCR templates complimentary to the SARS virus genome, which has the advantage of working on PCR templates without concern about viral infection and also has the advantage that it can be used by those who do not have access to the SARS virus. This highly efficient and safe strategy for obtaining SARS gene fragments is useful for the development of PCR assays, as well as for the preparation of reliable positive controls for PCR testing kits.

Index Entries: Gene synthesis; SARS; PCR, sequential primer extension.

1. Introduction

Since the initial completion of the SARS genome sequenced by a Canadian group on April 13, 2003 (1), a few polymerase chain reaction (PCR) protocols have been developed from several independent laboratories (2–4). However, a clinically oriented PCR diagnostic assay with high efficiency and reliability is not yet available. Although the worldwide outbreak of SARS has caused the loss of about one thousand lives (5), most research laboratories that have molecular or genetic expertise in China, and perhaps in other areas of the world, do not currently have access to the SARS virus owing to official regulation. The potential risk of an outbreak from experimental failure also limited the effort in developing a diagnostic assay for SARS.

In the present study, we describe a new strategy for *de novo* synthesis of deoxyribonucleic

acid (DNA) fragments complimentary to the SARS genome. By using a set of partially overlapping oligonucleotides, specific templates were quickly obtained by sequential primer extension. This strategy is simple and safe with no requirement for sophisticated equipment and does not need a highly regulated experimental environment. In addition to its urgent application in the development of the SARS diagnostic assay, this new strategy can also be used in the synthesis of genes, *in vitro*, for expression studies, especially for mutagenesis studies.

2. Material and Methods

2.1. Design of Overlapped Primer Set

Two sets of primers, set A and set B, synthesized by Sangon (Shanghai, China) were used in the sequential primer extension. Each of the sets contained three forward primers and one reverse

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Table 1
Sequences of Primers Used in Sequential Primer Extension

Primers	Sequence
Set A	
AF1	tgataagagctacgagcaccagacacccttcgaaattaagagtccaagaatttgaca <u>ctttcaagggaatgcc</u>
AF2	<u>ctttcaagggaatgcc</u> caaaagttgtttcctcttaactcaaaagtc <u>aaagtcattcaaccacg</u>
AF3	<u>aaagtcattcaaccacg</u> tgttgaagaaaagactgagggttcaggggc <u>tatacgctctgtgt</u>
AR	CAG GGT <u>ACA CAG AGC GTA TAC GCC C</u>
Set B	
BF1	attgtctctggttactggctacaacaatgtctttcgcttaaaagggggtgcaccaatta <u>aaggtgtaaccttggag</u>
BF2	<u>aaggtgtaaccttggaga</u> agatactgtttgggaagttcaaggtacaagaatgtgagaat <u>cacatt tgagcttgat</u>
BF3	<u>atcacatttgagcttgat</u> gaacgtgttgacaagtgcttaataagtgctctgtct <u>acactgttgatccgggtac</u>
BR	ACT TCG <u>GTA CCG GAT TCA ACA GTG T</u>

Note: AR and BR are reverse primers that were the starting points for the sequential extension. The underlined bases represent the portions of the sequences that were overlapped.

primer. All primers within one set were partially overlapped (**Table 1**). The first forward primer and the reverse primer of each set were chosen after being blasted within the Genbank to assure that these were conserved regions among the SARS strains so far identified and to avoid potential nonspecific products.

2.2. Sequential Primer Extension

Primer extension conditions are described elsewhere in detail (6). Briefly, primer extension was carried out for 30 cycles with 30 s denaturation at 95°C, 1 min annealing at 56°C, and 1 min extension at 72°C. The primer extension reaction was performed in a total volume of 25 µL with no template, 0.2 mM deoxynucleotide 5'-triphosphate (dNTP), 10 pmol/mL of both sense and antisense primers, 2.5 µL of the 10X NEB polymerase reaction buffer (New England Biolab, Beverly, MA), and 0.625 U *Taq* polymerases (Takara, Dalian, Liaoning). PCR products were visualized using 2% agarose gel electrophoresis running under 10 V/cm. Two types of sequential primer extension were performed: single-step sequential primer extension and consecutive-step sequential primer extension.

2.2.1. Protocol 1. Single-Step Sequential Primer Extension

All four primers in primer set A, designed for the production of Temp-I, or primer set B, de-

signed for Temp-II, were added to one reaction system and a different concentration for each primer was used in this sequential primer extension assay. The final concentration of the first forward primers of AF1 and BF1 and the reverse primers of AR and BR was 10 µM; the concentration of the second forward primers of AF2 and BF2 was 0.5 µM, and the concentration of the third forward primers of AF3 and BF3 was 0.025 µM. This single-step sequential primer extension was expected to yield mainly final templates with no intermediate products.

2.2.2. Protocol 2. Consecutive-Step Sequential Primer Extension

The number of separate reactions in consecutive-step sequential primer extension is as many as the number of forward primers. Step 1 uses a reverse primer together with the most 3' of the forward primers. Thereafter, the product of each reaction serves as the reverse primer for the next step. A method similar to this was described by Horton et al. (7). In this manner, the double-stranded products propagate from reverse primer toward the forward primers bridged by the overlapped sequences. As shown in **Table 2**, in the initial steps, forward primers AF3 or BF3 and the reverse primers AR or BR were the only oligonucleotides within the reaction system and were used at a final concentration of 10 µM. For all the

Table 2
Corresponding Products From Each Step of the Sequential Primer Extension

Sequential primer extension	Double strand converted	Expected products ^a	Product size (bp)
Set A			
Step 1	AF3_AR	...tcatg <u>gggcgtatacgctctgt</u> gtaccctg3'	74
Step 2	AF2_AF3—AR	...aaaag <u>tcaagtcattcaaccac</u> gtgtt	123
Step 3	AF1_AF2—AF3—AR	...5' tgaca <u>cttcaaggggaatgcc</u> caaag	182
Set B			
Step 1	BF3_BR	...tgtct <u>acactgttgaatccggtagcgaagt</u> 3'	83
Step 2	BF2_BF3—BR	...tgaga <u>atcacatttgagcttgat</u> gaacg	142
Step 3	BF1_BF2—BF3—BR	...5' aatta <u>aaggtgtaacccttggagg</u> aagat	204

^aThe expected products were extended from reverse primers (AR or BR) to the first forward primers. Each step extended over one primer bridged by the underlined overlapped sequences. As designed, the overlapped sequences in single-stranded primers next to each other were used as a bridge to convert the single-stranded primers into double-stranded products in which the underlined sequences were flanked by other sequences.

following steps, 1 μL PCR product from the immediate previous reaction was added to the reaction system containing the relevant forward and reverse primers.

2.3. Application of the De Novo Synthesized Templates in PCR Test

Two pairs of primers targeting the *de novo* synthesized templates were used. The primer pairs of AF with the sequence of 5'cgctctgataagagctacgagc3' and AR (see **Table 1**) targeted Temp-I. And the primer pairs of BF with the sequence of 5'-ctgcgcattgtctcc tggttactg3' and BR (see **Table 1**) were used to target Temp-II. The *de novo* synthesized templates were gel purified with a DNA fragment purification kit (Takara, Dalian), and the concentrations were measured by ultraviolet (UV) spectrometry. The templates were then serially diluted by a fraction of 10 using nuclease-free H₂O. PCR was performed in a total volume of 25 μL with the same conditions described in **Subheading 2.2.** after an initial denaturation of 2 min at 95°C.

3. Results

As shown in **Figure 1**, DNA fragments in lengths of 182 and 204 bp were obtained from primers of set A and set B, respectively, by the single-step sequential primer extension where each reaction contained four partially overlapping

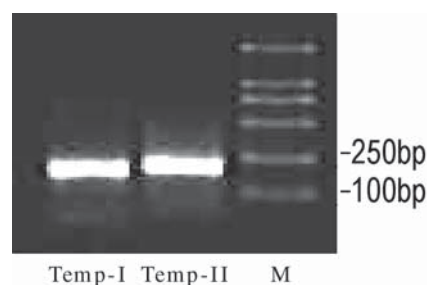


Fig. 1. A predominant single band was produced from the four primers of either primer set when the specified concentrations of the different primers were used. Temp-I was the product from primer set A and Temp-II was the product from primer set B. Intermediate products from steps 1 and 2 were difficult to detect because of the decreased amount of relevant primers used. *M* represents DNA marker.

primers. The products of the single-step sequential primer extension were the same size as predicted in **Table 2**, equaling the summation of the four overlapping primers minus half of the overlapping nucleotides.

When consecutive steps of sequential primer extension were used, each step yielded a product equal to the size of the two partially overlapping primers minus half of the overlapping bases. The

product of the next round reaction was always longer than the previous round. The longest desired products were those from the last steps, which were the final products of Temp-I and Temp-II (Fig. 2). The size of the final products was exactly the same whether they were produced from single-step or consecutive-step sequential primer extension. The consecutive-step sequential primer extension was used in case there were some primer sets in the single-step sequential primer extension that did not work. Sequence analysis confirmed that these two *de novo* synthesized templates were identical to the appropriate regions of SARS genome.

These two *de novo* synthesized templates were further evaluated in PCR reactions using different amounts of template. As shown in Figure 3, the templates obtained from the *de novo* synthesis worked well in testing primer pairs that targeted them. For reactions using 30 PCR cycles and 1000 or more copies of template, both the primer pair AF and AR and the primer pair BF and BR produced significant amount of PCR products. The amount of PCR product produced in these reactions correlated positively with the number of template copies used (Fig. 3).

4. Discussion

PCR reactions are widely used in both basic and clinical research, such as in the diagnosis of certain genetic and infectious diseases. The technological characteristics of PCR itself require the inclusion of both negative and positive controls, particularly when used in clinical applications. It is relatively simple in the diagnostic application in genetic diseases, whereas a positive control for the PCR kit used in infectious disease is more complicated and usually under stricter regulation. In the present study, we have reported a quick and safe strategy for *de novo* synthesis of DNA fragments complementary to the SARS viral genome. The *de novo* synthesized DNA fragment can be used in the development of PCR diagnostic assays and in clinical PCR testing kits as a positive control. The *de novo* synthesis of known genes of interest can also be used in preparation for expres-

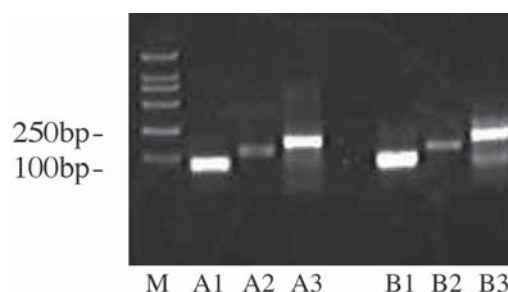
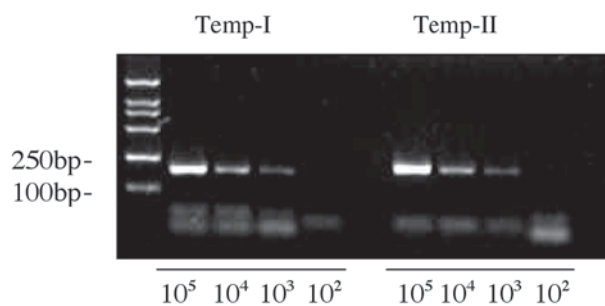


Fig. 2. An increasing size of products with increasing steps during consecutive-step sequential primer extension. *A1*, *A2*, and *A3* represent the products from steps 1, 2, and 3, respectively, for primer set A; *B1*, *B2*, and *B3* represent the products from steps 1, 2, and 3, respectively, of the sequential primer extension for primer set B. *M* represents DNA marker.



Copy number of templates employed

Fig. 3. Varying amount of PCR products amplified from different copy numbers of templates Temp-I and Temp-II. The amount of PCR products was significantly less when 100 copies of templates were used as compared with the products yielded from templates with 1000 or more copies. The two sets of primers showed similar sensitivities using the *de novo* synthesized PCR templates.

sion constructs for some specific proteins. In addition, the *de novo* synthesis of gene fragments using oligonucleotide sets is a straightforward strategy for mutagenesis studies.

Alternative methods for the production of templates are available but each has its own disadvantages. Chemical synthesis is rapid and routinely used for short oligonucleotides such as those shorter than 100 nucleotides. However, direct synthesis of long DNA fragment is technically lim-

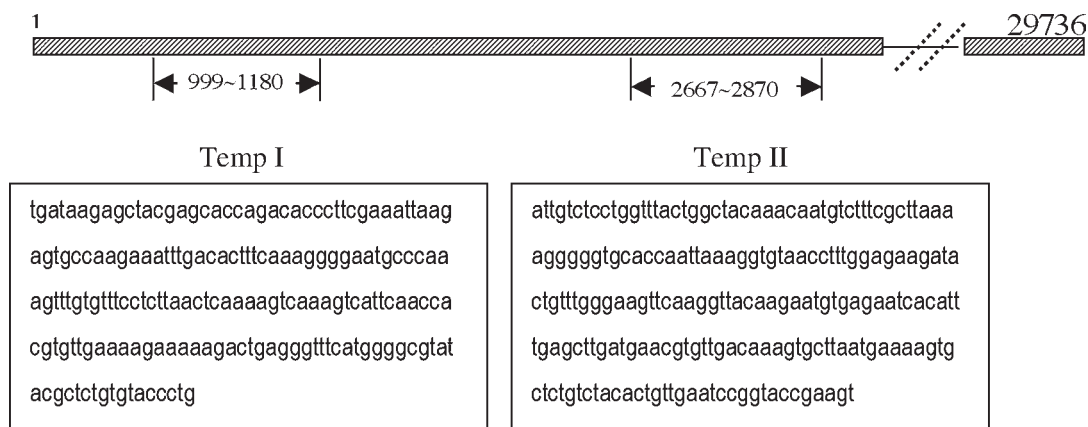


Fig. 4. Two gene fragments complementary to SARS virus TOR2 were obtained using the *de novo* gene synthesis technology. The Temp-I and Temp-II were complementary to 999-1180 and 2667-2870 of the SARS virus genome, respectively, as illustrated.

ited because the chemical reaction of each round is incomplete, thus the efficiency decreases with the propagation of reaction cycles. Taking advantage of chemical synthesis of oligonucleotides of fewer than 100 bases, the present strategy converted a set of partially overlapping, short, single-stranded oligonucleotides into a longer, double-stranded segment, simply by employing a new technology for the *de novo* synthesis of genes or gene fragments, sequential primer extension.

It is possible to chemically synthesize a set of double-stranded fragments with overhanging terminals compatible with each other and then to combine them by sequential ligation. Theoretically, both the sequential ligation and the sequential primer extension work for *de novo* synthesis of gene fragments. We did not test the sequential ligation strategy, as it is more expensive and time consuming. The urgent need in SARS diagnostic assay encouraged us to use sequential primer extension.

The outbreak of SARS demonstrates the need for rapid response to unknown diseases in the future. *De novo* synthesis of the PCR template complementary to a viral genome provides a tool for the rapid development of early diagnostic assays for any new pathogen as soon as its sequence is known. For example, the two *de novo* synthesized DNA fragments described here can serve as positive controls in clinical PCR tests because these

two fragments are designed from the complementary sequences of SARS coronavirus TOR2 (*I*) as shown in **Figure 4**. Although the sequential primer extension was developed for SARS diagnostic assay, the technology itself has wide applications in other fields in functional genomics. For instance, the *de novo* synthesis of a mutated, truncated, or chimeric gene encoding a specific protein will be possible and is very useful.

Taken together, the *de novo* synthesis of the PCR template reported in the present study provides a simple and quick technology in preparing a gene fragment of interest. The sequential primer extension is not technically difficult as compared to routine PCR, except for its requirement of a set of overlapping primers. The templates developed from this new method can be directly used as positive control in PCR assay. It is hoped that a wide application of the *de novo* synthesis of the PCR template will facilitate the development of a clinical PCR kit for the early diagnosis of SARS.

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