

A Nonspecific Primer Anchored PCR Technique for Chromosome Walking

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ABSTRACT

*A chromosome walking method was improved in this work. The new method was named as nonspecific primer anchored PCR (NPA-PCR). Nested gene specific primers were designed based on the known region and long random primer using degeneracy oligonucleotides for nonspecific anchoring. Annealing temperatures were varied to control the priming. Target sequences were obtained by PCR with random primer and gene-specific primer. Nonspecific sequence with long random primers at both ends formed stem loop structure due to inverted terminal repeats. The method was employed to isolate a gene with newly-isolated actinomycin producing strain *Streptomyces setonii* Z-L-22. A 0.77 kb fragment of actinomycin synthetase gene cluster was isolated from the strain. The fragments of 1474bp and 701bp were obtained, respectively, at the up and down streams of known fragment through the this method. NCBI Blast analysis showed that the walking sequence and the known sequence were located conjointly in the same cluster gene. It demonstrated that the result was correct and this technique could be useful and efficient for chromosome walking or isolating the gene.*

Key words: nonspecific primer anchored PCR; chromosome walking; random primer

INTRODUCTION

The hunt for missing sequence data is often required in the experiments. Several polymerase chain reactions (PCR)-based methodologies are available for walking from a known region to cloned or uncloned genomic DNA, including inverse PCR (IPCR) (Triglia et al., 1988; Melanie et al., 2004, Ochman et al., 1988), adaptor ligation (LM-PCR) (Espelund and Jakobsen, 1992; Kilstrup and Kristiansen, 2000; Yan et al., 2003; Chen et al., 2004) and randomly primed PCR (RP-PCR). The first two methods have high specificity

but require enzyme digestion and ligation, making them relatively expensive and time-consuming. Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995) is a representative method of the third one. This method has its specific advantage but the amplified products are generally limited to a length of <1 kb.

Here we report a nonspecific primer anchored PCR (NPA-PCR), which has been developed on the basis of Peng's (Peng, 2005) nonspecific anchoring of suppression sequence PCR (NASS-PCR).

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MATERIALS AND METHODS

Template DNA

Streptomyces setonii Z-L-22 was used in this work. Its genomic DNA was extracted according to the method described by Kieser et al., (2000).

Walking primers

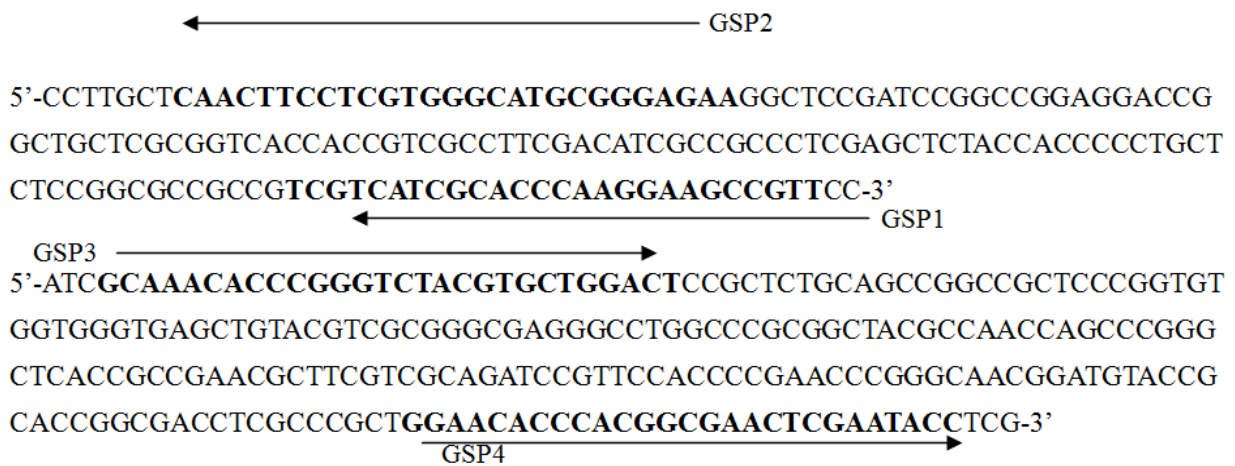
Two sets of nested specific primers were designed

SAP1 5'-CCTGAGACCTATTACCGCCCAGAGGATCTCCGTGTNNNTGCAT-3'

SAP2 5'-CCTGAGACCTATTACCGCCCAGAGGA-3'

SSP1 5'-GTAATACGACTCACTATAGGGCTGAGACGCATGATACCTCTGCAT-3'

SSP2 5'-GTAATACGACTCACTATAGGGC-3'



PCR procedure

Four steps were included in NPA-PCR. In the first step, PCR mixture (15 µl final volume) including 50 ng of template DNA, 1 U of long and accurate *Taq* DNA polymerase (TaKaRa Biotechnology Co., Ltd), 0.2µM dNTP mixture, 10 µl 2 × GC buffer I, 0.1µM SAP1. 5µl GSP1 (with the final concentration of 0.5µM) was added, and the reaction cycles were run (Table 1) then the second step started. The third step was carried out in 25 µl volume of reaction mixture composed of the reaction products above and 2.5µl SAP2 with the final concentration of 0.5µM and 2.5µl 2 × GC buffer I and run the program in table 1. For the fourth step, 1µl of the third step PCR products diluted 50 times with Milli-Q water was used as the PCR template. The reaction mixture was composed of 0.5 µM GSP2, 0.5 µM SAP2, 0.2 M

based on the known sequence. GSP1 and GSP2 started respectively from 216bp and 89bp in the upstream of the known region. GSP3 and GSP4 started respectively from 502bp and 701bp in the downstream of the known region. The sequence of anchoring primers SAP1, SAP2 used in NPA-PCR and SSP1, SSP2 used in NASS-PCR together with gene specific primers are shown as follows.

dNTPs, 1 × GC buffer I and 1 U *Taq* DNA polymerase. After the PCR, the reaction mixture was resolved by electrophoresis on a 1.2% agarose gel and DNA fragments were visualized by staining in ethidium bromide. Reactions of NPA-PCR and NASS-PCR are shown in Table 1.

Cloning and sequencing of the PCR product

The PCR products were purified from agarose gel with DNA purified Kit (TaKaRa Biotechnology Co., Ltd) and linked with pMD19-T vector following the introductions given in the Kit. The plasmid was transformed into *E. coli* JM109 competent cells. The positive clones were sequenced by Shanghai Sangon Biological Engineering Technology and Services Company. DNA sequence analyses were carried out using the BLAST program (<http://ncbi.nlm.nih.gov>).

Table 1 - Reactions of NPA-PCR and NASS-PCR.

NPA-PCR				NASS-PCR			
steps	Primers adding	cycles	procedure	steps	Primers adding	cycles	procedure
1	SAP1	1	94°C 5min	1	SSP1 GSP1 (GSP3)	1	94°C 5min
			30°C 3min			10	94°C 30s
			ramp to 65°C				60°C 1min
			at the rate of 0.25°C/min			1	72°C 3min
2	GSP1 (GSP3)	10	94°C 30s	2	SSP2	1	94°C 30s
			65°C 3min				60°C 1min
		1	72°C 5min				ramp to 65°C
3	SAP2	1	94°C 2min	3	GSP2 (GSP4) SSP2	1	94°C 2min
		20	94°C 30s			30	94°C 30s
			65°C 3min				60°C 1min
		1	72°C 5min			1	72°C 7min
4	GSP2 (GSP4) SAP2	1	94°C 2min	1	SSP2	1	94°C 2min
		30	94°C 30s			30	94°C 30s
			65°C 3min				60°C 1min
		1	72°C 5min			1	72°C 7min

RESULTS

The principle of NPA-PCR

The principle of NPA-PCR is illustrated in Figure 1. Four primers were used in NPA-PCR. Two sequence anchoring primer (SAP), SAP1 and SAP2 (a part of SAP1), were as the nonspecific anchoring. Two gene specific primers (GSP), GSP1 and GSP2, were nested primers (Fig. 1a). Four steps were included in NPA-PCR. First, a single cycle of PCR was carried out at a low annealing temperature with only primer SAP1. At this temperature, SAP1 could prime at target or non-target positions on the DNA template (Fig. 1b). Then the annealing temperature was increased to the point corresponding to the annealing temperature of GSP1. GSP1 was added at this time the amplification products were produced when SAP1 hybridized with target position by SAP1 and GSP1, meanwhile the non-target gene amplification was suppressed by the stem-loop structure of the DNA (Fig. 1c). SAP2 was added in the third step. The amplification was carried out by GSP1 and SAP2 to generate the template (Fig. 1d). The products of third step were diluted and used as template in the last step. Target DNA was exponentially amplified by the nested PCR with GSP2 and SAP2 (Fig. 1e). NPA-PCR and NASS-PCR were carried out according the protocol described above. The PCR products were separated on 1.2% agarose gel and

examined (Fig. 2). The amplification results were the same for the upstream walking by NPA-PCR and NASS-PCR. Bands with the length of approximately 1500bp were obtained. Different results were obtained at downstream walking: a band of approximately 700bp by NPA-PCR while 600bp by NASS-PCR. The products were purified and sequenced.

Sequence analysis

Upstreamed 1474bp-fragments were obtained both by NPA-PCR and NASS-PCR. The sequences were the same except for the primer sequences of SAP1 and SSP1. The walking sequences had 86bp overlap with known sequence. A fragment of 701bp was obtained by NPA-PCR at the downstream with 70bp overlap to the known one and was consistently with design. A 2716bp-fragment obtained by assembling the walking sequence and the original sequence was shown below. The sequence had 79% identity with actinomycin synthetase III (acmC) gene cluster of *Streptomyces chrysomallus*, which was consistent with the antibiotic kind of *S. setonii* Z-L-22. A downstream 641-bp fragment was obtained at the end by NASS-PCR. The sequence with SSP1 at both ends showed no overlap with the known one and no similarity with any other sequences by NCBI blast analysis. It should be non-specific amplification sequence.

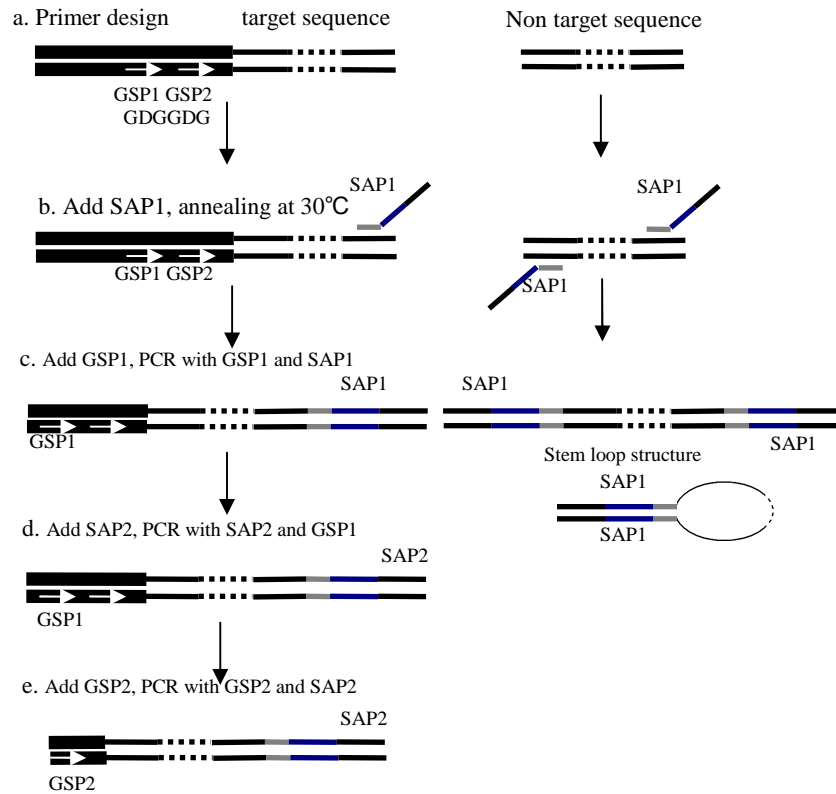


Figure 1 - Schematic outline of NPA-PCR method for chromosome walking. Known and unknown sequences are depicted with thick and thin lines, respectively. DNA sequences with thin white arrows in them represent the same sequences as the primers. SAP1 is composed of grey, blue and black segments. Grey segments show the expected specific hybridized sites, black segments show the same part of SAP1 and SAP2, blue segments show the nonspecific hybridized parts particular in SAP1.

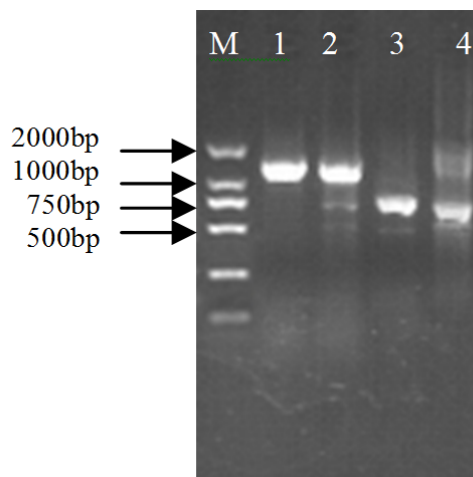


Figure 2 - Amplification results of genomic DNA of *Streptomyces setonii* Z-L-22 by NPA-PCR and NASS-PCR. M, DNA ladder: DL2000. 1: upstream walking by NPA-PCR; 2: upstream walking by NASS-PCR; 3: downstream walking by NPA-PCR; 4: downstream walking by NPA-PCR.

CCTGAGACCTATTACCGCCCAGAGGATCTCCGTGTCCTCTGCATAACGAACTCCTCGGCGA

SAP1

CCAGAACGACCCCCACAGCCTCTTCACCAACCAGATCACCCACTGGAAAAACACCCTCGAA
 AACCTCCCCGACCACATCACCATCCCCACCGACCGACCCCGCCCCACCATCACCACCTACCA
 AGGCGACTACACCACCATCACCATCAACCCACCCCTCCACCAAAAAATCACCCACCTCGCCC
 ACACCACCAACACCACCGTCTTCATGGTCCTCCAAGCAGGACTCACCCACCTCCTCACCAAG
 CTCGGCGCCGAAACGACATCCCCATCGGCACCCCATCGCCGGACGCACCGACCACAACC
 TCGACAACCTCATCGGCTTCTTCGTCAACACCCTCGTCCTCCGCACCGACACCACCAACAAC
 CCCACCTTACCCAACTCATCAACCGCATCCGCGAAACCAACCTCACCGCCTACGCCACCA
 AGACGTCCCCTTCGAATACCTCGTCGAAATCCTCAACCCACCCGCACCTCAACCACCACC
 CCTTCTCAAATAATGCTCGCCCTCCAAAACGCACCCGAAGGAGCATTCCAACTCCCCGGC
 CTCACCATCGACATCGCCCCGGACGCACCGGAACCGCAAATTCGATCTCTTCTTCAGCCT
 CGTCGAGAAACGCGGACCCACGGCGAACCCGAAGGCATCACCGGCGCCATCGAATACTCC
 AGCGACCTCTACGACGAAACCACCGTCCACACCCTCTTCGAACGCCTCACCCGCATCCTCGA
 AACCGCCACCGACACCCCGACCAACCCCTCAGCCACATCGACGCCCTCAGCCCGCACGAG
 AAGCACCAGGTGCTGGACTCCTGGCTGGAGACGGCGACGGAGGTAGGGGCGGTCTGCTGC
 CTGCCCGCTTCTCAGAGCAAGCGTCCGCGACACCGGACGCCGTGTCCCTGGTCGCCGGCGAC
 ACCGTCTGACCTATGCCGAACTGGACCGGCGGGCCAACCGGCTGGCCCCGAGTCTGCTCGA
 CCGAGGTGCGGGAGCCGCGCTGTGACCGCCATCGCGCTGCCGCGCTCGGCCGACCTGGTG
 GTGGCACTGCTGGCAGTGCTCAAGTCCGGCGGGCCTACCTGCCGCTCGACCCCGACCACCC
 GCGCGCACGCCTTACGCACGTCTTGAGGATGCCCGCCCTCACTGCTGCTCACGACCACGG
 CGACGGACTGGATGATCCCGGAGGTGGGCGCCGCACAGCGGCTGGTCCTCGATTCCGACCC
 CGTACGGGAGGCATCGGACGCCGCCCTCGACAGCGATCCGGCCGACGACGGCGGGGTGCGC
 CCCCTCCGGCCGGAGGACGCCGCGTACGTATCTACACCTCGGGCTCGCACCGGCCGGCCGA
 AGGGCGTGGTGGTCCCGCACGGCGCCTTGCTCAACTTCCTCGTGGGCATGCGGGAGAAGGCT
 CCGATCCGGCCGGAGGACCGGCTGCTC

GSP2

GCGGTCACCACCGTCGCCTTCGACATCGCCGCCCTCGAGCTCTACCACCCCTGCTCTCCGGC
 GCCGCCGTCGTCATCGCACCCAAGGAAGCCGTTCCGCAGCCCTCGGCCGTGCTGGACCTGAT
 CGAACGGCACAGCGTCACGGTCGTGCAGGGCACCCCGTCCCTGTGGCAACTGCTCGTGGCGC
 ACGACGCGGAGGCGCTTCGAGGGCTACGGATACTGGTCGGCGGCGAAGCGCTGCCGGTCTC
 CCTCGCCGAGGCCCTGCGCGGGCTCACCGACGACCTGGTCAACCTGTACGGCCCCGACCGAG
 ACCACCATCTGGTCGACCGCTGCGGATCTGGCCGGCCACACCGGAGGGCCGGCACCGATCG
 GGCGGCCGATCGCAAACACCCGGGTCTACGTGCTGGACTCCGCTCTGCAGCCGGCCGCTCCC
 GGTGTGGTGGGTGAGCTGTACGTGCGGGCGAGGGCCTGGCCCCGCGGCTACGCCAACAGC
 CCGGGCTCACCGCCGAACGCTTCGTGCGAGATCCGTTCCACCCCGAACCCGGGCAACGGATG

TACCGCACCGGCGACCTCGCCCGCTGGAACACCCACGGCGAACTCGAATACCTCGGCCGTAC
CGA

GSP4

CCACCAGGTCAAGATGCGGGGCTACCGCATCGAACCCGGCGAGATCGAGAAGACCCTCACC
GACCACCCCGACATCGCCAGGCCGCGGTGATCGTACGGGAGGACAGGCCGGGAGACCTCC
GCCTGGTCGGGTACGTCGTGGCCGACAGCGGGGGCGGAGTCCGTGACGAGCATGTTCGAGCG
GGACCAGCTGAGCGAGTGGCAGGACCTCTACGACTCCGTCTACACGTCCGCCGGCGAAACT
GACATCGGCGAGAACTTCGCGAGCTGGAACAGCAGTTACGACGGTCAGCCCATCCCCTGG
AAGACATGCGGGAATGGCGCGACAGCACCGTCGACCGCATCAAAGCACTCCACCCACGACG
CGTCCTGGAAATCGGCGTTCGGCACCGGCCCTCTGCTCGCCAAAATCGCACCCGGACTGCGACG
AATACTGGGGAACCGACTTCTCCCCGACCGTCATCGAAGACCTACGACACCACATAGAAGC
CGACCCCCACCTCACCGAGAAAGTCACCTCCGCACCCAAGCCGCACACGAACACGGCGAC
CTCAAGAAGAACCCTTCGACACCATCATCCTCAACTCCGTCGTGCAGTACTTCCCCAACGC
CGGATGCATGGAGCTGCCTCTAGGAGACCCGCCATTATCCAGAGTCC

SAP1

Amplification of the assembling sequence from genome DNA

The PCR were carried out using the gene specific primers designed from the assembling sequence with genome DNA to verify the walking results.

The amplification results are shown in Figure 3. Expected fragments were 2100bp and 1300bp. The amplification fragments shown in the gel were consistent with the expected. Sequencing results were same as the expected.

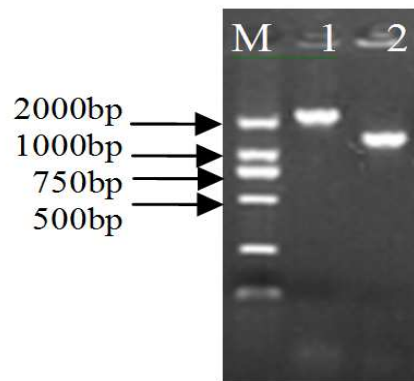


Figure 3 - Validation the walking results by amplification M, DNA ladder marker, DL2000. 1: Validation the assembling sequence at 5' by amplification; 2: Validation the assembling sequence at 5' by amplification.

DISCUSSION

Anchored PCR is often used for the amplification of cDNA ends due to the same homology oligonucleotide at 3' ends of mRNA (Ohara et al., 1989; Frohman et al., 1988) and generally could get good results. However, the DNA structures are not same, anchored PCR can't be used in DNA without any manipulation. Nonspecific anchoring primer, equal to adding the same structure to DNA, was used in this work, which could accomplish the amplification of unknown region. Chromosome walking methods based on the random primer is well accepted due to simplicity and specialty (Jiang et al., 2007; Tan et al., 2005). The NPA-PCR method described in this work was designed on the basis of NASS-PCR. Random primers were redesigned and reactions were improved. NPA-PCR showed a higher specialty when employed in chromosome walking in *Streptomyces setonii*. The method was based upon the principle that a primer may initiate PCR at any sequences that had only partial homology to the 3' end. Chromosome walking was accomplished by using two gene specific primers (GSP) which annealed to the known sequence, and a nonspecific anchoring primer (SAP1) which had sequences to be complement to a particular region that were not known near the target sequence. SAP1 hybridized to multiple sites on both the coding and complementary strands of template DNA. Targeted PCR products would only be produced when SAP1 primer annealed to a DNA strand that was contiguous with and complementary to the strand of DNA to which gene specific primer had hybridized.

SAP1, a partially degenerate primer, plays a key role in the process. It bears the effect of priming, anchoring by nonspecific hybridization and suppressing amplification of non-target sequence. The oligonucleotides 5'-NNNNNTGCAT-3' at the 3' end of SAP1 were utilized to find the TGCAT sites on the template molecules at low temperature with the help of random nucleotides NNNNN (Tan et al., 2005). Non-target molecule, amplified by single primer SAP1, contained SAP1 sequences at both the ends. The ends of the single non-target strands formed a stem-loop structure when annealing during every cycle in the second step. Stem-loop structures, stable than the primer-template hybrid, suppressed the amplification. The length of SAP1 (44bp) facilitated the formation of PCR suppression effect.

For SAP1, as random primer, the length and kind of oligonucleotide could be varied. Different number and kinds of specific anchoring oligonucleotides at 3' ends of SAP1 would lead to different amplification results. Five specific oligonucleotides were chosen in this work and a sequence of 1474bp was obtained in upstream chromosome walking in *S. setonii* Z-L-22. Analyzing the sequence between SAP2 and GSP2, TGCAT sites appeared once thus creating one clear band in the gel electrophoresis. There would be more TGCAT sites of contiguous DNA far from the target sequence, which wasn't be amplified due to the length. To be addressed, two aspects increasing the specificity should be noticed in NPA-PCR in the second step. First, the concentration of GSP1 should be low while the concentration of SAP1 should be high (Wang et al., 2007). Second, GSP1 should be added at its annealing temperature.

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