

Annealing control primer system for improving specificity of PCR amplification

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A novel primer designed to improve the specificity of PCR amplification, called the annealing control primer (ACP), comprises a tripartite structure with a polydeoxyinosine [poly(dI)] linker between the 3' end target core sequence and the 5' end nontarget universal sequence. We show that this ACP linker prevents annealing of the 5' end nontarget sequence to the template and facilitates primer hybridization at the 3' end to the target sequence at specific temperatures, resulting in a dramatic improvement of annealing specificity. The effect of this linker is demonstrated by the incorporation of ACP sequences as primers during the amplification of target nucleotide sequence and as hybridization probes in the genotyping of single nucleotide polymorphisms. This is the first report to show that a poly(dI) linker between two different sequences of ACP forms a bubble-like structure and disrupts or destabilizes DNA duplex formation at certain annealing temperatures.

INTRODUCTION

The success of PCR amplification relies solely on the specificity with which a primer anneals to its target sequences. Therefore, it is important to optimize this molecular interaction (1). The annealing temperature is critical for determining whether a primer binds only to its perfect complement or to sequences with one or more mismatches. By adjusting the annealing temperature, one can alter the specificity of pairing between template and primer. Numerous approaches have been introduced to increase primer annealing specificity. Longer primers with universal, homopolymer, or loop sequence tails at their 5' ends improve the specificity of PCR amplification and the stability of hybridization

(2–4). However, this approach does not abrogate nonspecific hybridization resulting from the involvement of the tail or loop sequence in the priming reaction. This limitation of longer primers necessitates the specification of the annealing sequences of the primers to exclude nonspecific annealing. Here we describe a novel annealing control primer (ACP) that specifically targets sequence hybridization to the template via a polydeoxyinosine [poly(dI)] linker. The structure of ACP comprises (i) a 3' end region with a target core nucleotide sequence that substantially complements the template nucleic acid for hybridization; (ii) a 5' end region with a nontarget universal nucleotide sequence; and (iii) a poly(dI) linker bridging the 3' and 5' end sequences.

Rationale of Annealing Control Primer Oligonucleotides

The presence of universal bases in a primer, such as deoxyinosine (5,6), 1-(2'-deoxy- β -D-ribofuranosyl)-3-nitropyrrole (7), and 5-nitroindole (8), leads to lower annealing temperatures due to weaker hydrogen bonding interaction in base pairing. In this study, we show that the presence of a poly(dI) linker between the 3' end and 5' end sequences of a primer generates a region with a lower melting temperature (T_m) via the formation of a bubble-like structure at specific temperatures. The poly(dI) linker in the bubble-like structure affects the annealing of each region of the primer. Essentially, the ACP linker prevents the 5' region from annealing under conditions in which the target 3' sequence anneals to the template at the original annealing temperature.

MATERIALS AND METHODS

First-Strand cDNA Synthesis

Total RNAs (Seegene, Seoul, Korea) from 18.5-day-old mouse conceptus tissues were isolated and used for the synthesis of first-strand cDNA by reverse transcriptase as previously described (9). The cDNA synthesis primer sequence was dT-ACP1 (5'-CGTGAATGCTGCGACTACGATII-IITTTTTTTTTTTTTTTTTT-3'), in which the poly(dT)₂₀ at the 3' end was replaced as poly(dT)₁₈.

Amplification of a RIKEN cDNA Target Sequence

First-strand cDNA was synthesized from mouse conceptus total RNA [18.5 days postcoitus (dpc)] using dT-ACP1 as specified above. A 635-bp fragment

of RIKEN full-length cDNA (accession no. NM 027815) (RIKEN, Saitama, Japan) was amplified using conventional primers or ACP (Table 1). A two-stage amplification reaction was conducted in 50 μ L reaction mixture comprising 50 ng first-strand cDNA, 5 μ L 10 \times buffer (Promega, Madison, WI, USA), 5 μ L 25 mM MgCl₂, 5 μ L dNTP mixture (2 mM each), 1 μ L 5' primer (10 μ M), 1 μ L 3' primer (10 μ M), and 0.5 μ L *Taq* DNA polymerase (5 U/ μ L) (Promega). We used the following PCR conditions: two cycles of 94° for 1 min, 50° for 3 min, and 72° for 1 min; followed by 40 cycles of 94° for 40 s, 65° for 40 s, 72° for 40 s, and a 5-min final extension cycle at 72°C. The amplified products were observed on 2% agarose gel.

Amplification of a Short Fragment Containing a p53 Single Nucleotide Polymorphism

A 349-bp fragment containing a single nucleotide polymorphism (SNP) in exon 4 of the human p53 gene was amplified using human genomic DNA templates and the following primers, P53N-ACP (5'-TATGAATGCTGTGACGCCGAIIII-CCTCTGACTGCTCTTTTCAC-3') and P53C-ACP (5'-TCACAGAAGTATGCCAAGCGAIIIIATTTGAAGTCTCATGGAAGCC-3'). DNA templates were obtained from human blood samples containing an SNP of mutant homozygotes in exon 4 of *TP53* (the tumor protein p53 gene). PCR was performed in a 50- μ L reaction mixture comprising 50 ng genomic DNA, 5 μ L 10 \times PCR buffer, 5 μ L 25 mM MgCl₂, 5 μ L dNTP mixture (2 mM each), 1 μ L P53N-ACP (10 μ M), 1 μ L P53C-ACP (10 μ M), and 0.5 μ L *Taq* DNA polymerase (5 U/ μ L). The PCR conditions used were 30 cycles of 94° for 40 s, 65° for 40 s, and 72° for 40 s, followed by a 5-min final extension cycle at 72°C. Amplified products were purified using a QIAquick® PCR purification kit (Qiagen, Valencia, CA, USA) and employed as probes in allele-specific dot blot hybridization.

Allele-Specific Dot Blot Hybridization

Oligonucleotides (6 μ L of 100 μ M) were spotted on a Hybond™ N⁺ membrane (Amersham Biosciences, Piscataway, NJ, USA) and immobilized using

an optimized UV cross-linking procedure. Initial hybridization was conducted using the radioactively labeled 349-bp fragment in 10 mL QuikHyb® solution (Stratagene, La Jolla, CA, USA) at 40°C for 2 h. The hybrid was washed 3–5 times with a buffer solution of 2 \times standard saline citrate (SSC) and 1 \times SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature for 1–10 min and exposed to film at -80°C. The resultant membrane filter used in the first step was re-employed for a second hybridization without stripping to remove the probe used in the first hybridization. The second hybridization step was conducted using the first hybridized filter and [γ -³²P]-labeled oligonucleotide comprising a sequence complementary to the second hybridization region of longer conventional or ACP oligonucleotides in 10 mL QuikHyb solution at 58°C for 2 h. The hybrid was washed three times with a buffer solution of 2 \times SSC and 1 \times SSC/0.1% SDS at 60°C for 5 min and exposed to film at -80°C. The radiation dose of each dot was measured by autoradiography to evaluate the strength of the hybridization.

RESULTS AND DISCUSSION

To determine whether the poly(dI) linker of ACP prevents annealing of the 5' region under conditions in which the 3' region is fully hybridized to the template

at the first annealing temperature, the amplification of a RIKEN cDNA target sequence was compared using ACP and longer conventional primers. Both ACP and longer primers comprise 10-mer target sequences at their 3' ends and nontarget universal sequence tails at their 5' ends (Table 1). Consistent with our expectations, a pair of longer conventional primers with the same structure as ACP [except for the presence of the poly(dI) linker] generated many nonspecific products, including the target product (Figure 1, lane 2). Upon replacing the 5' sequences of the conventional primers with other nontarget universal sequences, different patterns of nonspecific products were generated, as observed on a 2% agarose gel (Figure 1, lanes 3, 6, and 7). In contrast, a pair of ACP primers produced the expected target product (Figure 1, lanes 5, 10, and 11, confirmed by cloning and sequencing; data not shown). We further examined whether other common nucleotide residues [e.g., (dA)₅ or (dT)₅] would be applicable for constructing a polydeoxynucleotide linker by substituting deoxyinosine with polydeoxyadenosine [poly(dA)]. Primers with the poly(dA) linker generated nonspecific products, regardless of the nontarget universal sequences at the 5' regions (Figure 1, lanes 4, 8, and 9).

Three major conclusions can be drawn on the basis of these results. (i) Nontarget universal sequences of longer conven-

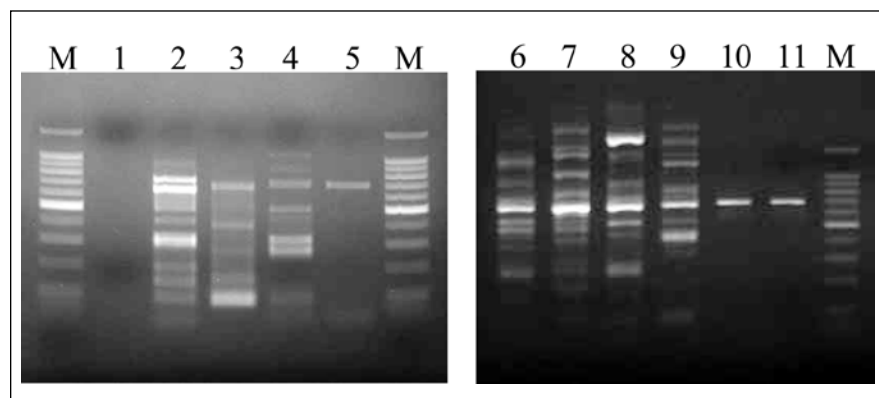


Figure 1. Amplification of a target RIKEN cDNA fragment using conventional primers or annealing control primer. Short conventional primers (such as 10-mers) used as a negative control produced no visible products, since their melting temperatures (T_m s) were 32° (5' primer) and 34° (3' primer), but the first annealing temperature was 50°C (lane 1). Primer combinations (5' to 3') are as follows: lane 1, 10-mer + 10-mer; lane 2, 10-mer-tail1 + 10-mer-tail2; lane 3, 10-mer-tail2 + 10-mer-tail1; lane 4, 10-mer-A₅-tail1 + 10-mer-A₅-tail1; lane 5, 10-mer-I₅-tail1 + 10-mer-I₅-tail1; lane 6, 10-mer-tail1 + 10-mer-tail3; lane 7, 10-mer-tail3 + 10-mer-tail1; lane 8, 10-mer-A₅-tail1 + 10-mer-A₅-tail3; lane 9, 10-mer-A₅-tail3 + 10-mer-A₅-tail1; lane 10, 10-mer-I₅-tail1 + 10-mer-I₅-tail3; lane 11, 10-mer-I₅-tail3 + 10-mer-I₅-tail1. M represents the 100-bp marker (Promega).

Table 1. Oligonucleotides Used in this Study

Primers Used in the Amplification of a RIKEN cDNA Target Sequence	
5' Primer	Sequence
10-mer	5'- AGGAGATGCG -3'
10-mer-tail1	5'-GTCTACCAGGCATTGCTTCAT AGGAGATGCG -3'
10-mer-tail2	5'-TATGAATGCTGTGACGCCGA AGGAGATGCG -3'
10-mer-tail3	5'-CTGTGAATGCTGCGACTACGAT AGGAGATGCG -3'
10-mer-A ₅ -tail1	5'-GTCTACCAGGCATTGCTTCAT <u>AAAAA</u> AGGAGATGCG -3'
10-mer-I ₅ -tail1	5'-GTCTACCAGGCATTGCTTCAT <u>IIIIII</u> AGGAGATGCG -3'
10-mer-A ₅ -tail3	5'-CTGTGAATGCTGCGACTACGAT <u>AAAAA</u> AGGAGATGCG -3'
10-mer-I ₅ -tail3	5'-CTGTGAATGCTGCGACTACGAT <u>IIIIII</u> AGGAGATGCG -3'
3' Primer	Sequence
10-mer	5'- GGTCACGGAG -3'
10-mer-tail1	5'-GTCTACCAGGCATTGCTTCAT GGTCACGGAG -3'
10-mer-tail2	5'-TATGAATGCTGTGACGCCGAG GGTCACGGAG -3'
10-mer-tail3	5'-CTGTGAATGCTGCGACTACGAT GGTCACGGAG -3'
10-mer-A ₅ -tail1	5'-GTCTACCAGGCATTGCTTCAT <u>AAAAA</u> GGTCACGGAG -3'
10-mer-I ₅ -tail1	5'-GTCTACCAGGCATTGCTTCAT <u>IIIIII</u> GGTCACGGAG -3'
10-mer-A ₅ -tail3	5'-CTGTGAATGCTGCGACTACGAT <u>AAAAA</u> GGTCACGGAG -3'
10-mer-I ₅ -tail3	5'-CTGTGAATGCTGCGACTACGAT <u>IIIIII</u> GGTCACGGAG -3'
Oligonucleotides Used in Allele-Specific Dot Blot Hybridization^a	
<i>TP53</i> (Exon 4)	5'-ATGAAGCTCCAGAATGCCAGAGGCTGCT CCCC [G/C] <u>CGTG</u> GCCCCCTG-3'
1. Short (10-mer)	5'- TCCCC [G/C] <u>CGTG</u> -3'
2. Longer (3 additional matches)	5'-GTCTACCAGGCATTGCTT <i>TGCT</i> CCCC [G/C] <u>CGTG</u> -3'
3. Longer (2 additional matches)	5'-GTCTACCAGGCATTGCTT <i>CGCT</i> CCCC [G/C] <u>CGTG</u> -3'
4. Longer (1 additional match)	5'-GTCTACCAGGCATTGCTTCA <i>CT</i> CCCC [G/C] <u>CGTG</u> -3'
5. Longer (No additional matches)	5'-GTCTACCAGGCATTGCTTCAT CCCC [G/C] <u>CGTG</u> -3'
6. AC (3 additional matches)	5'-GTCTACCAGGCATTGCTT <i>GAG</i> <u>IIIIII</u> TCCCC [G/C] <u>CGTG</u> -3'
7. AC (No additional match)	5'-GTCTACCAGGCATTGCTTCAT <u>IIIIII</u> TCCCC [G/C] <u>CGTG</u> -3'
8. A5 (No additional match)	5'-GTCTACCAGGCATTGCTTCAT <u>AAAAA</u> TCCCC [G/C] <u>CGTG</u> -3'
9. Probe for 2nd hybridization	3'-CAGATGGTCCGTAAGCGAAGTA-5'
<p>The bold letters represent the target sequences (10-mer) for annealing or hybridization. The polydeoxynucleotide linkers are underlined. I represents deoxyinosine.</p> <p>^aA partial sequence of the human <i>TP53</i> (exon 4) spanning polymorphic bases was aligned with the oligonucleotide sequences used in this study. Polymorphic bases are depicted in brackets [wild-type/mutant]. Additional matched nucleotides immediately upstream of the target sequence are italicized.</p>	

tional primers are involved in primer annealing, which results in numerous nonspecific products. (ii) Nontarget universal sequences of ACP are not involved in annealing while the 3' target regions anneal to the template during first-stage PCR, resulting in specific products only. (iii) Linkers composed of other polydeoxynucleotides [e.g., (dA)₅ or (dT)₅] do not have the same benefits as the poly(dI) linker. Thus, our data clearly show that the poly(dI) linker of ACP facilitates the targeting of the 3' sequences to be hybridized to the template under stringent conditions. Although a two-stage PCR

is applied to both ACP and longer primer systems to increase annealing specificity, the longer primers are not free from the problems caused by the involvement of nonspecific tail or loop sequences in primer hybridization (2–4). In contrast, the ACP system utilizing the two-stage PCR prevents the nonspecific sequences from annealing to the template due to the effects of the poly(dI) linker, resulting in a significant increase in annealing specificity. This is the first report to describe the role of the poly(dI) linker in distinguishing between two different annealing sequences of PCR primers in association

with annealing temperature.

The key conditions and parameters of this technique for the amplification of a target sequence are as follows. The first annealing temperature should be 10°–20°C higher than the T_m of the 3' target core sequence of the ACP. The length of the 3' end target sequence is designed to have a T_m ranging from 30°–55°C, and shorter primer sequences (between 10- and 15-mers) are more compatible for maximizing the advantage of the ACP system, where the GC content is dependent on the length of the 3' end target sequence and its T_m. Further, the other

PCR parameters, such as buffer and salt concentration, depend on the DNA polymerase used in the reaction.

To determine the optimum length of the linker, ACP sequences were examined by varying the number of deoxyinosine residues in the linker. Optimum function was observed at a length of five bases (data not shown). Accordingly, the five-base linker was used throughout the experiments described in this report.

The utility of the poly(dI) linker of ACP in PCR applications was additionally confirmed by evaluating its effects on a single base mismatch. We compared the discriminatory effects of ACP and conventional oligonucleotides on SNPs. Oligonucleotides were immobilized on a nylon membrane. A region containing an SNP in exon 4 of *TP53* was amplified using ACP. This polymorphism (10) is expressed as an Arg to Pro substitution at position 72 (as a result of replacing G with C). A 349-bp sequence of the *TP53* gene was amplified from mutant homozygotes.

Surprisingly, the 10-bp 3' target sequence of allele-specific ACP oligonucleotides effectively discriminated between perfectly matched and one base

mismatched duplexes (Figure 2, rows 6 and 7). In contrast, longer allele-specific conventional oligonucleotides failed to distinguish between the two duplex types (Figure 2, rows 2–5). The poly(dA) linker did not function in the same manner as the poly(dI) linker (Figure 2, row 1), which is consistent with data from PCR applications (Figure 1). These results further support our hypothesis that the poly(dI) linker of ACP oligonucleotides specifically targets the hybridization sequence to the 3' allele-specific region under conditions of the first hybridization step, resulting in SNP discrimination.

Furthermore, regardless of the presence of three additional complementary nucleotides in the 5' sequence, the poly(dI) linker of ACP oligonucleotides prevents this region from hybridizing to the template (Figure 2, row 6). The same phenomenon is evident in ACP-based PCR amplification.

Shorter, conventional oligonucleotides did not form hybrids under such high-stringency conditions (Figure 1, row 1). In contrast, ACP and longer conventional oligonucleotides formed hybrids with mutant genomic DNA fragments under these conditions (Fig-

ure 2, rows 2–8). Our results imply that the additional tailing sequences of both longer conventional and ACP oligonucleotides increase the strength (i.e., efficiency) of the first hybridization region, thus allowing higher hybridization and washing temperatures. Our results are consistent with a spacer effect proposed by Saiki and co-workers (3).

To confirm the results of the first annealing step, a second step was conducted using the resultant membrane filter employed in the first step without stripping and a labeled oligonucleotide probe complementary to the nontarget universal sequence of ACP primers. The results of the second hybridization step showed that each spot had almost equal radioactivity (Figure 2). These data suggest that the spotting and immobilization of the oligonucleotide probes were conducted equally in the membrane and that allele-specific ACP primers function effectively as probes for the discrimination of a single base mismatch in the first hybridization stage. Accordingly, the quality of the first hybridization can be verified through the second step.

Interestingly, the poly(dI) linker of ACP oligonucleotides affected not only the first hybridization sequence (3' region) but also the second hybridization sequence (5' region). Longer conventional oligonucleotides were not sufficiently sensitive to detect mismatched bases in the second hybridization sequence and thus failed to distinguish between a perfect match and a three-base mismatch (Figure 2). However, ACP oligonucleotides displayed a much weaker signal for mismatched bases in hybridization sequences than for a perfect match (Figure 2). These results suggest that the poly(dI) linker induces hybridization specificity for both 3' and 5' sequences of ACP oligonucleotides. An additional benefit of the structure of ACP oligonucleotides is that a rational universal sequence applicable to multiple templates or genes can be designed because the poly(dI) linker allows for the second sequence to hybridize separately from the first sequence, which is extremely difficult with longer conventional oligonucleotides.

In conclusion, the evaluation of ACP oligonucleotides as primers or probes in the amplification of target nucleotide sequences and SNP discrimination reveals the potential of tripartite structure

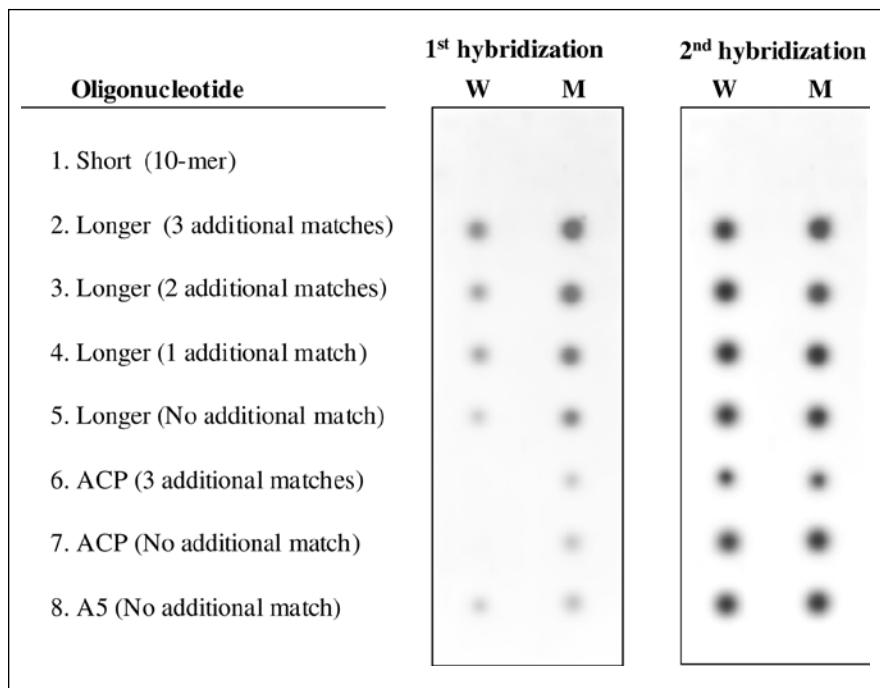


Figure 2. Allele-specific dot blot hybridization for distinguishing a single base mismatch. Allele-specific short (row 1), longer (rows 2–5 and 8), and annealing control primer (ACP) (rows 6 and 7) oligonucleotides were immobilized on the membrane. Longer and ACP oligonucleotides have tail sequences at their 5' end with additional matched nucleotides (0–3) immediately upstream of the target sequence as indicated. W, wild-type oligonucleotide; M, mutant-type oligonucleotide.

oligonucleotides to increase the hybridization strength of target sequences in all fields of nucleic acid amplification and hybridization-based methods.

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