

## Polymerase Chain Reaction with Confronting Two-pair Primers for Polymorphism Genotyping

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**A novel PCR method using confronting two-pair primers, named PCR-CTPP, is introduced to detect a single nucleotide polymorphism (base X or Y). One primer for the X allele is set to include X' at the 3' end (antisense), where X' is the antisense of X, with the counterpart sense primer upstream. For the Y allele, a sense primer including Y at the 3' end is set, with the antisense primer downstream. One common band and one specific band for each allele are amplified, which allows genotyping directly by electrophoresis. This method is exemplified by application to the polymorphisms of beta-adrenoceptor 2 and interleukin 1B. It is simpler than PCR-RFLP (restriction fragment length polymorphism), which requires incubation with a restriction enzyme, and is suitable for genotyping in studies of genetic epidemiology involving hundreds of samples.**

Key words: PCR — Confronting two-pair primers — Polymorphism

Studies on the associations between genotypes and disease risk have been increasing rapidly and provide useful information to resolve biological mechanisms and to develop prevention strategies.<sup>1–3</sup> In order to obtain a stable estimate of the strength of an association, a large sample size is required. In recent studies on gene-environment and/or gene-gene interactions,<sup>4,5</sup> hundreds of subjects needed to be genotyped. Accordingly, there is a demand for a quick genotyping technique at a low cost. For the genotyping of single nucleotide polymorphisms (SNPs) by polymerase chain reaction (PCR),<sup>6</sup> PCR-RFLP (restriction fragment length polymorphism), DNA sequencing, and PCR-SSCP (single-strand conformation polymorphism)<sup>7</sup> are now available. DNA microarrays are also applicable,<sup>8</sup> but the method is very expensive at present. In studies of genetic epidemiology with a large sample size, PCR-RFLP has become the most common method for genotyping, if applicable, because of its low cost and technical easiness.

PCR-RFLP involves three steps; PCR with a thermal cycler, incubation with a restriction enzyme for PCR product digestion, and electrophoresis for visualizing the genotype. The second step (incubation) requires 3 to 24 h, depending on the restriction enzyme. If this step could be skipped, as in PCR genotyping for VNTR (variable number of tandem repeats) polymorphisms, the study would be much quicker to conduct. This paper introduces a novel method named PCR-CTPP (confronting two-pair primers),

which allows genotyping for SNPs without PCR product digestion, and further, for SNPs without restriction sites.

The logic of PCR-CTPP can be explained as follows for a single nucleotide polymorphism of base X or Y. The antisense nucleotide for X is denoted as X'. As depicted in Fig. 1, confronting pairs of primers (four primers in all) are used; one pair is for the X allele and the other is for the Y allele. One primer for the X allele (antisense primer 1 R) is set to include X' at the 3' end, with the counterpart (sense primer 1 F) upstream. Correspondingly, a sense primer including Y (primer 2 F) and an antisense primer (primer 2 R) downstream of primer 2 F are set for the Y allele. The sizes of the DNA products to be amplified specifically for each allele are *a* bp and *b* bp, respectively. The sizes, *a* and *b*, must be sufficiently different for the products to be distinguishable by electrophoresis on gel. For XX genotype, *a* and *c* bp bands are observed, because the DNA sequence between primers 1 F and 2 R is also amplified, but not the sequence between primers 2 F and 2 R, while for YY genotype, *b* and *c* bp bands are observed, where  $c = a + b - (d - 1)$ , and *d* is the sum of the sizes of primers 2 F and 1 R, which surround base X/Y. A heterozygous genotype shows three bands, *a*, *b*, and *c* bp.

Study subjects were outpatients who have participated in one of the studies run at Aichi Cancer Center since 1999 (Ethical Committee Approval Number 12-20, 12-23, 12-27).<sup>9,10</sup> A 7 ml peripheral blood sample was drawn after written informed consent had been obtained, including permission to examine gene polymorphisms. DNA was extracted from buffy coat fractions with a Qiagen

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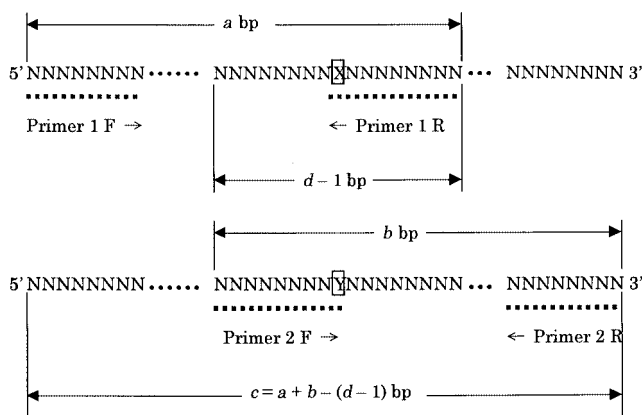


Fig. 1. Logic of polymerase chain reaction with confronting two-pair primers (PCR-CTPP). *a*, size for X allele; *b*, size for Y allele; *c*, size between primers 1 F and 2 R; *d*, sum of the sizes of primers 2 F and 1 R.

QIAamp DNA Blood Mini Kit (QIAGEN Inc., Valencia, CA).

Two SNP examples are shown in this paper. The first is a C-to-G (Gln27Glu) polymorphism of beta-adrenoceptor 2 (BAR-2), which has been reported to be related to obesity and/or metabolic disorders.<sup>11)</sup> The primers for the C

allele were 5'-CCG CTG AAT GAG GCT TCC-3' (sense) and 5'-CCA CAC CTC GTC CCT TTG-3' (antisense), and those for the G allele were 5'-ACC ACG ACG TCA CGC AGG-3' (sense) and 5'-GGC TGT GAT GAC CAG CAC-3' (antisense). The G/C proportion of the primers was 61.1% (11/18) or 66.7% (12/18). The sequence around the selected primers (Accession No. NM000024) is shown in Table I. Genomic DNA (30 to 100 ng) was used in a 25- $\mu$ l reaction mixture with 0.15 mM dNTPs, 25 pmol of each primer, 0.5 units of "Takara Taq" (Takara Shuzo Co., Ltd., Otsu), 2.5  $\mu$ l of 10 $\times$  PCR Buffer including 15 mM MgCl<sub>2</sub> (Takara Shuzo Co., Ltd.), and 1  $\mu$ l of glycerol. Amplification conditions were 5 min of initial denaturation at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C, and a 5 min final extension at 72°C. PCR products were visualized on a 2% agarose gel with ethidium bromide staining. Genotyping was performed as follows; 279, 204 bp for CC genotype, 279, 204, 110 bp for CG genotype, and 279, 110 bp for GG genotype.

The second example is a C-to-T polymorphism at -31 of interleukin 1B (IL-1B), which was reported to increase the risk of stomach cancer.<sup>12)</sup> The primers for the C allele were 5'-ACT TCT GCT TTT GAA GGC C-3' (sense) and 5'-TAG CAC CTA GTT GTA AGG A-3', and those for the T allele were 5'-AGA AGC TTC CAC CAA TAC T-3' (sense) and 5'-CTC CCT CGC TGT TTT TAT A-3' (anti-

Table I. DNA Sequences for Beta-adrenoceptor 2 (BAR-2) and Interleukin- 1B (IL-1B)

BAR-2 (Accession No. NM000024)	101	CCACACCACA	<u>GCCGCTGAAT</u>	<u>GAGGCTTCCA</u>	GCGTCCGCT
			C allele sense primer →		
	281	ACCACGACGT	CACGCAG <u>C</u> AA	AGGGACGAGG	TGTGGGTGGT
			← C allele antisense primer		
BAR-2 (Accession No. NM000024)	281	ACCACGACGT	CACGCAG <u>G</u> AA	AGGGACGAGG	TGTGGGTGGT
			G allele sense primer →		
BAR-2 (Accession No. NM000024)	371	ATGTGCTGGT	<u>CATCACAGCC</u>	ATTGCCAAGT	TCGAGCGTCT
			← G allele antisense primer		
IL-1B (Accession No. X04500)	1791	CCTCCCCTAA	<u>GAAGCTTCCA</u>	<u>CCAATACTCT</u>	TTTCCCCTTT
			T allele sense primer →		
	1881	TCCTACTTCT	GCTTTTGAAA	G <u>C</u> TATAAAAA	CAGCGAGGGA
			← T allele antisense primer		
IL-1B (Accession No. X04500)	1921	<u>GAAACTGGCA</u>			
	1881	TCCTACTTCT	GCTTTTGAAA	G <u>C</u> TATAAAAA	CAGCGAGGGA
			C allele sense primer →		
IL-1B (Accession No. X04500)	2020	TCCTTACAAC	<u>TAGGTGCTAA</u>	GGGAGTCTCT	CTGTCTCTCT
			← C allele antisense primer		

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

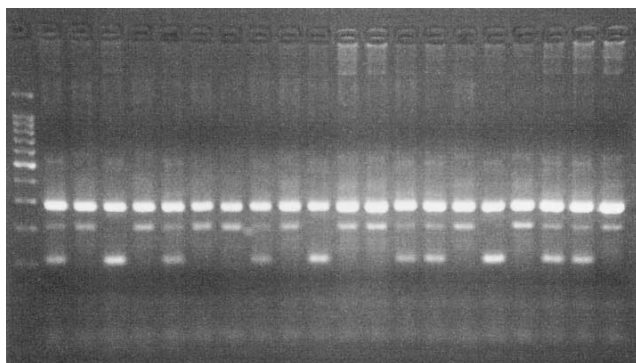


Fig. 2. Gel showing the genotype for a C-to-G (Gln27Glu) polymorphism of beta-adrenoceptor 2. Lane M contains a 100 bp DNA ladder, lanes 2, 4, 6, 7, 9, 11, 12, 15, 17 and 20 the C/C genotype (279, 204 bp), lanes 1, 5, 8, 13, 14, 18 and 19 the C/G genotype (279, 204, 110 bp), and lanes 3, 10 and 16 the G/G genotype (279, 110 bp).

sense). The G/C proportion of the primers was 42.1% (8/19) or 47.4% (9/19). The PCR mixture components were the same as before, except that glycerol was not added. The amplification conditions were 5 min of initial denaturation at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and a 5 min final extension at 72°C. The sequence around the primers (Accession No. X04500) is shown in Table I. Genotyping was distinguished as follows; 240, 155 bp for CC genotype, 240, 155, 122 bp for CT genotype, and 240, 122 bp for TT genotype.

The results are shown in Figs. 2 and 3. Clear bands were obtained by the PCR-CTPP method. All of the samples showed the same genotype as that determined by PCR-RFLP methods. In the case of BAR-2 (Fig. 2), 10 samples showed CC genotype, 7 showed CG genotype, and 3 showed GG genotype, and in the case of IL-1B (Fig. 3) 3 showed CC genotype, 8 showed CG genotype, and 9 showed GG genotype.

There is no doubt that PCR-CTPP is a time-saving, cheap method. In addition, in terms of the possibility of genotyping misclassification, this method is probably superior to PCR-RFLP. PCR-CTPP involves four different primers, instead of two. However, the PCR-CTPP method skips the digestion step, so it is free from errors related to the preparation of restriction enzyme mixture. If PCR is not successful, or if primers 1 F or 2 R fail to be mixed, the *c* bp band is not observed. Thus, technical errors are easy to identify. This method is applicable to polymorphisms for which an appropriate restriction enzyme is not available, as well as for to insertion or deletion polymorphisms. On the other hand, primers with a similar melting temperature have to be used. If a suitable primer set can

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 M

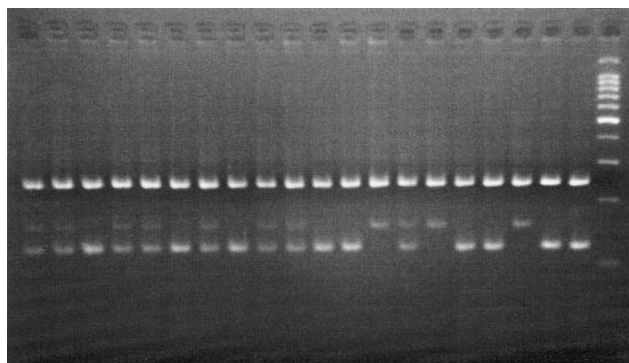


Fig. 3. Gel showing the genotype for C-to-T polymorphism at -31 of IL-1B. Lane M contains a 100 bp DNA ladder, lanes 13, 15 and 18 the CC genotype (240, 155 bp), lanes 1, 2, 4, 5, 7, 9, 10, and 14 the CT genotype (240, 155, 122 bp), and lanes 3, 6, 8, 11, 12, 16, 17, 19 and 20 the TT genotype (240, 122 bp).

not be found, this method is not applicable. This is a common problem to usual PCR with one pair of primers.

To our knowledge, this is the first paper to describe this simple method and its application for actual genotyping. Blum *et al.* compared PCR-RFLP with allele-specific amplification of N-acetyltransferase polymorphisms.<sup>13)</sup> Their primers for different alleles were set in the same direction, not in the opposite direction like ours. They conducted PCR separately with one pair of primers, so the basic idea was different. Takeda *et al.* reported a method, MASA (mutant-allele specific amplification), to detect a mutation by using multiple primers paired to one counterpart primer, which is also different from our method.<sup>14)</sup> Studies using duplex or triplex PCR have been reported,<sup>15)</sup> but their purpose is not to skip the digestion step. The idea of genotyping dependent on the different sizes of products generated by two pairs of primers is new.

This method has the advantage of great simplicity, and is also applicable for detection of somatic mutations, so it should be useful for genetic diagnosis of cancer. When PCR-CTPP is applicable, it should be superior to PCR-RFLP. We have already shifted to this method to save time and cost for genotyping. DNA microarrays will eventually be cheaper, but may not be so effective for genotyping of a limited number of SNPs with a large sample size. Our method should have a role in genotyping for sometime.

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