CCR: a rapid and simple approach for mutation detection

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ABSTRACT

We describe a simple approach for detecting known mutations in genomic DNA. The strategy entails a DNA amplification reaction that combines the use of thermostable DNA polymerase and ligase, and that has been designated the Combined Chain Reaction (CCR). CCR consists of four phases: denaturation, annealing, elongation and ligation. Unlike most PCR-based mutation detection systems it relies on mismatch between primer and template at the primer 5′ ends. It is rapid and simple, and requires neither the use of radioactivity, nor polyacrylamide gel electrophoresis, nor autoradiography for mutation detection at the single base-pair level.

The polymerase chain reaction (PCR) allows the amplification of a discrete segment of DNA from a large pool of irrelevant DNA sequence (1). Among its first applications was the detection of the known nucleotide substitution associated with sickle cell anemia (2). Since that time, PCR has revolutionized and become an essential tool for molecular biology. It is now used for diagnosis of hereditary and infectious diseases, routine DNA amplification, site-directed mutagenesis, cloning of genomic DNAs and cDNAs, DNA sequencing, DNA fingerprinting and forensic science. The Ligase Chain Reaction (LCR), was developed as an alternative amplification procedure and has been used for detecting known point mutations (3,4) and for introducing mutations into DNA in vitro. To combine the applications of PCR and LCR, we describe a DNA amplification strategy that utilizes both thermostable DNA polymerase and thermostable ligase in a single reaction. The designation for this combined chain reaction is CCR. Each CCR cycle consists of four steps: denaturation of template DNA, annealing of primers to single-stranded templates, elongation of primers by DNA polymerase, and ligation of the 3′ OH end of an extended primer with the 5′-phosphate end of a downstream primer.

The underlying principle of the CCR approach is illustrated in Figure 1. Like LCR, CCR requires four primers for mutation detection, but the primers are positioned differently. Two external primers flank the overall sequence that is amplified while two internal diagnostic primers have their 5′ ends positioned at the putative mutation site. The differential ability to form a phospho-diester bond between perfectly matched ends and mismatched ends is the basis of LCR, and is also the basis for CCR in this application and is described in detail in the legend to Figure 1.

To test the applicability and efficiency of CCR for detecting known mutations in genomic DNA, a known mutation at P53 in HaCaT cells was analyzed. The HaCaT cell line is derived from a human squamous cell carcinoma (5), and is a compound heterozygote at P53 with two different mutant P53 alleles (6). The mutation targeted for detection was a dinucleotide substitution CC→TT at codon 281–282 in exon 8. Genomic DNA from the human fibrosarcoma cell line HT1080 was used as a control since this cell line lacks mutations in P53 within exons 5–9 (7). The external primer EP1 was positioned at nt 14 106–14 125 and the other external primer EP2 was placed at nt 14 804–14 785 on the other strand (Fig. 2a). The internal primers extended from nt 14 512 to 14 535 and from 14 513 to 14 489. The internal primers MU and ML match the mutant P53 sequence; whereas the internal primers WU and WL match the wild-type P53 sequence. A third primer pair, CU and CL, that matched neither, was used as a control. When BCR was applied to HT1080 genomic DNA, the wild-type internal primers produced the predicted 699 bp fragment in addition to the two smaller 293 and 408 bp fragments (Fig. 2b). The internal, mutant primers produced only the smaller fragments since the mismatched 5′ ends are not ligated with sufficient efficiency to produce the 699 bp fragment. It is highly improbable that the two small fragments will be ligated in subsequent cycles because the 5′ terminal nucleotides of each pair of diagnostic primers are complementary. In preceding cycles, an extra 2 nt will have been generated at the junction site further reducing the likelihood of ligation. This reduces background when diagnostic primers are mismatched with template. In contrast to HT1080 cells, HaCaT cells have one allele mutated at that site. Thus, the diagnostic, full length fragment was produced using either the MU/ML or WU/WL primer pairs, but not using the control primer pairs.

For most practical applications, the sensitivity of mutation detection is paramount. Using 50 ng of genomic HaCaT DNA, which is equivalent to ~900 copies of P53 genomic DNA or ~450 copies of each of the HaCaT P53 alleles, CCR produced a detectable signal indicative of mutant DNA. With 25 ng, the signal was detectable but weak. While the conditions might be optimized to produce a stronger signal, an alternative strategy was implemented. A larger fragment of the target sequence first was

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amplified using the primers p53P1 and p53P2 which are complementary to nt 14 086–14 106 and 14 969–14 950 respectively, and which are located outside primers EP1 and EP2. The 884 bp amplification product was diluted 10 000-fold into a reaction mix containing primer pairs EP1/EP2 for nested CCR, together with the appropriate internal primers. As shown in Figure 2b, the amplification products were the same as when genomic DNA was used directly, but the amplification signal was significantly improved with lower background.

To further assess the reliability and reproducibility of this method, the large ligated fragments produced by primer sets EP1/EP2 and WU/WL (Fig. 2b, lane 4, lower panel) and EP1/EP2 and WU/WL (Fig. 2b, lane 5, lower panel) were recovered, digested, amplified and reamplified as before. Large ligated fragments were obtained only with internal primers WU and WL for the wild-type sequence recovered from lane 4, and only with internal primers MU and ML from the mutant DNA recovered from lane 5. This result illustrates the sensitivity and fidelity of the approach since only wild-type sequence was amplified with wild-type primers and only mutant sequence was amplified with mutant primers. The data also indicate that the procedure is reliable and reproducible, and that nanogram quantities of genomic DNA are sufficient for analysis.

To demonstrate that CCR can detect single nucleotide mismatches, preamplified HT1080 P53 genomic DNA was reamplified with internal primer sets that had only a single nucleotide mismatch at their 5′ ends. As shown in Figure 3, (lanes 2–4) a single 5′ base substitution is sufficient to eliminate production of the diagnostic large fragment, indicating that CCR can efficiently detect missense and nonsense mutations in genomic DNA.

Application of CCR to mutation detection requires that the thermostable DNA polymerase lack 5′→3′ exonuclease activity. If present, this activity can remove the mismatched 5′ end of the internal primers producing fully complementary hybrids with matched ends that serve as good ligation substrates producing the diagnostic large fragment. Also, a 5′→3′ exonuclease activity can potentially displace the extended downstream primer, producing an artificial large fragment. The polymerase used in these experiments was native Pfu DNA polymerase which has 3′→5′ activity but lacks 5′→3′ exonuclease activity. The 3′→5′ exonuclease activity is not required for mutation detection, but can be advantageous in that its proof-reading activity increases...
polymerase fidelity (8). Although the 3′→5′ exonuclease activity is desirable for polymerase fidelity, it reduces processivity, which in turn can limit the size of the fragment generated.

The CCR strategy for mutation detection differs from that of PCR or LCR. For PCR, mismatched nucleotides are at the 3′ end of the oligonucleotide primer. For LCR, the mismatches can be at either the 3′ or 5′ end. With CCR, mismatched nucleotides are restricted to the 5′ end of the primer, contributing to enhanced selectivity. The sensitivity of CCR for direct mutation detection is ~25 ng genomic DNA; however, preamplification of the target sequences significantly increased the level of sensitivity (Fig. 2b). The sensitivity should be further improved upon optimization of the reaction conditions with respect to buffer composition, pH, amount of ligase and polymerase, and parameters of the thermocycle. Further analysis of preamplified target sequences increases the sensitivity of the approach to the point that limiting tissue availability should not be an issue.

Allele-specific PCR, and its modifications is widely used for mutation detection (11). Differential amplification by allele-specific PCR utilizes two primers, one of which contains a mismatch at the 3′ end. Since base-pairing at the 3′ end is important for PCR amplification efficiency when Taq or other DNA polymerases which have no proof-reading activity are used, certain mismatches at the 3′ end can reduce amplification greatly. Although allele-specific PCR is quite robust, a shortcoming of allele-specific PCR is that it does not detect all known mutations. Some primers with mismatched 3′ ends still can permit efficient amplification. A single extension of a mispaired primer early in the allele-specific PCR reaction will result in an artifactual signal. Of 12 possible mismatches, four significantly reduce PCR yields while others do not (12). LCR has been shown to be able to detect some mismatches which were problematic with allele-specific PCR. In a comparative study, three mismatches, which could not be recognized by allele-specific PCR, were detected by LCR (4), suggesting that ligase is more sensitive to mismatch than polymerase. Since the basis by which CCR distinguishes mismatches from true matches is essentially the same as LCR, it is not surprising that CCR retains the high sensitivity to mismatches.

Two modified LCRs, repair chain reaction (RCR) (13) and gap-LCR (14) in which a thermostable DNA polymerase plays a key role, have been described for detecting known mutations. These methods rely on the differential ability of a polymerase to fill a small gap between adjacent oligonucleotides before ligation takes place to detect mutations. The RCR method requires different nucleotides (dCTP/dGTP or dATP/dTTP) to fill the gap, whereas gap-LCR, like allele-specific PCR relies on allele-specific elongation to fill the gap. In both cases, the extended oligonucleotides are ligated by thermostable ligase.

There are significant advantages to CCR. The first is its speed and its simplicity. The only required steps are DNA isolation, CCR amplification, agarose gel electrophoresis and product visualization. Second, the DNA amplified by CCR is easily detectable in agarose because of its relatively high molecular weight. The larger size is advantageous since it can be visualized by ethidium bromide staining and does not require primer labeling, acrylamide gel electrophoresis or autoradiography. Its simplicity lends itself to rapid screening, and reduces overall test cost.

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