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# Detection of point mutations with a modified ligase chain reaction (Gap-LCR)

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## ABSTRACT

**DNA amplification systems are powerful technologies with the potential to impact a wide range of diagnostic applications. In this study we explored the feasibility and limitations of a modified ligase chain reaction (Gap-LCR) in detection and discrimination of DNAs that differ by a single base. LCR is a DNA amplification technology based on the ligation of two pairs of synthetic oligonucleotides which hybridize at adjacent positions to complementary strands of a target DNA. Multiple rounds of denaturation, annealing and ligation with a thermostable ligase result in the exponential amplification of the target DNA. A modification of LCR, Gap-LCR was developed to reduce the background generated by target-independent, blunt-end ligation. In Gap-LCR, DNA polymerase fills in a gap between annealed probes which are subsequently joined by DNA ligase. We have designed synthetic DNA targets with single base pair differences and analyzed them in a system where three common probes plus an allele-specific probe were used. A single base mismatch either at the ultimate 3′ end or penultimate 3′ end of the allele specific probe was sufficient for discrimination, though better discrimination was obtained with a mismatch at the penultimate 3′ position. Comparison of Gap-LCR to allele-specific PCR (ASPCR) suggested that Gap-LCR has the advantage of having the additive effect of polymerase and ligase on specificity. As a model system, Gap-LCR was tested on a mutation in the reverse transcriptase gene of HIV, specifically, one of the mutations that confers AZT resistance. Mutant DNA could be detected and discriminated in the presence of up to 10 000-fold excess of wild-type DNA.**

## INTRODUCTION

The ability to detect single base changes is of great importance in molecular genetics. Specific identification of point mutations in the human genome plays a major role in diagnosis of hereditary diseases and in identification of mutations within oncogenes, tumor supressor genes and of mutations associated with drug resistance.

Single base variations have been analyzed by a variety of techniques, such as restriction fragment length polymorphism (1), denaturing gradient gel electrophoresis (2) and chemical cleavage of mismatched heteroduplexes (3). Other techniques include RNAse cleavage of mismatched bases (4) and single strand conformation polymorphism (5). All of these techniques have the advantage of being able to screen for unknown mutations. Yet, they are very labor intensive, multistep, non-automated processes and most importantly lack sensitivity (6). Recently, highly sensitive amplification-based techniques have been developed, among which are hybridization of allele-specific oligonucleotides to polymerase chain reaction (PCR)-amplified products (7,8) and competitive oligonucleotide priming, where differential amplification depends on differential hybridization (9). The amplification refractory mutation system (10), also referred to as allele-specific PCR (ASPCR) (11), which relies on positioning the mutation at the 3′ end of a PCR primer, and the ligase chain reaction (LCR), where a mismatch is positioned at the ligation joint (12–14), are two other amplification technologies used for analysis of single base mutations.

In the LCR, two pairs of synthetic oligonucleotides which hybridize at adjacent positions to complementary strands of a target DNA are joined by a thermostable ligase. Multiple rounds of denaturation, annealing and ligation result in the exponential amplification of the target DNA (Fig. 1A) (13–18). Targets that differ by a single base pair are discriminated, since a mismatch at the ligation joint severely reduces the efficiency of ligation (12–14,19). Generation of target-independent ligation products due to blunt-end ligation poses limitations on the sensitivity of LCR (20). Typically, the sensitivity of LCR or any diagnostic assay is not a critical factor for detection of mutations in human genetic diseases, where 50 or 100% of DNA contains the mutation. In contrast, for detection of somatic mutations within oncogenes, tumor supressor genes or drug resistance mutations, where a small number of mutated molecules need to be detected in the presence of excess wild-type DNA, sensitivity becomes a critical factor.

Several approaches have been taken to increase the sensitivity of LCR. One approach has been to use another amplification technology, such as PCR, followed by limited amplification with LCR (21,22). Other alternatives are PCR followed with the ligation detection reaction (LDR), where only two adjacent probes are used, resulting in linear amplification (13,14,20,21), or PCR followed with the oligonucleotide ligation assay (OLA),

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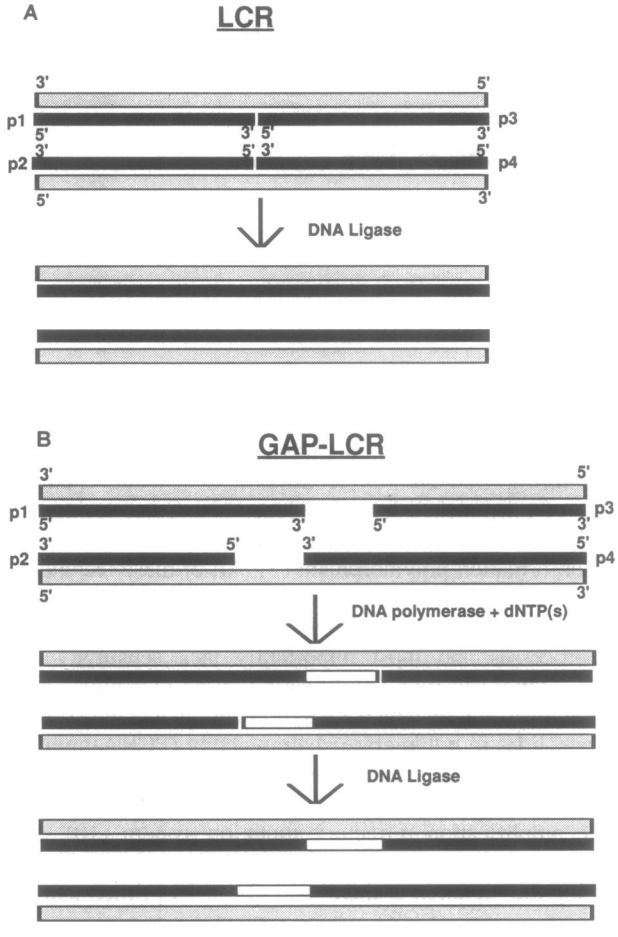


**Figure 1.** Diagrammatic representation of LCR and Gap-LCR. The complementary strands of target DNA are represented as shaded bars, LCR probes as solid bars and regions extended by DNA polymerase as white bars. (**A**) In LCR, four probes covering the entire target sequence anneal to complementary strands, probe 1 is ligated to probe 3 and probe 2 to probe 4 by a thermostable DNA ligase. The ligated probes function as targets in subsequent cycles and exponential amplification is achieved. (**B**) In Gap-LCR, probes 1 and 4 have 3′ overhangs with respect to their complements. Probes 1 and 4 are extended by DNA polymerase with the appropriate nucleotide(s) to fill the gap and ligated to probes 3 and 2 respectively with the ligase.

where ligation of two adjacent probes is used as a single detection step (12,19,23). However these combined approaches necessitate the opening of tubes after PCR, generating a source of contamination and also introducing complexity to automation.

A modification of LCR, Gap-LCR has been introduced to circumvent these difficulties and improve the sensitivity of LCR (24–27). In Gap-LCR, complementary probe pairs containing 3′ extensions are used. After hybridization to target DNA, a gap of one to several bases exists between adjacent probes. A thermostable DNA polymerase, devoid of 3′→5′ exonuclease activity, and the appropriate nucleotide(s) are used to fill the gap and the resultant probes are joined by DNA ligase (Fig. 1B). The use of probe duplexes with non-complementary 3′ extensions prevents the generation of target-independent ligation products. Gap-LCR has been successfully applied to detect < 10 target molecules in a reaction (unpublished results). Amplification products are detected by a sandwich immunoassay performed with an automated analyzer (17,26). The sensitivity, specificity and automation of the technology make Gap-LCR a good candidate for diagnostic tests.

In this study we explored the properties of Gap-LCR in the detection and discrimination of target DNA sequences that differ by a single base pair.

## MATERIALS AND METHODS

### Oligonucleotides and plasmids

Target DNAs (50 nt) were synthesized and gel purified by Genosys (The Woodlands, TX). The sequence of the wild-type target was derived from the sequence of the *Chlamydia trachomatis* cryptic plasmid, map position 2230–2280 (28). Mutant A and Mutant B targets were identical to the wild-type target, except single base changes were introduced at the indicated positions to both strands during synthesis (Fig. 2A). Targets for HIV experiments (Fig. 5) were gifts from Dr Steve Wolinsky (Northwestern University) and Dr John Mellors (University of Pittsburgh). They were provided as purified DNA from plasmids containing a 1.7 kb fragment of the HIV genome cloned into *Eco*R1 and *Hin*dIII sites of the vector pKK233 (Pharmacia). The mutant sequence has a mutation at amino acid 215 which changes the codon from ACC to TAC (from threonine to tyrosine) (Fig. 5A). The sequence of the region used as the target for LCR is 5′-AACATCTGTTGAGGTGGGGATTTACCACACACCAGACAAAAAACATCAGA. The LCR probe sets were synthesized on an Applied Biosystems synthesizer 394 by the phosphoramidite method. The 5′ end of probe 1 and 3′ end of probe 2 were covalently linked to carbazole, while the 3′ end of probe 3 and 5′ end of probe 4 were linked to adamantane (Fig. 2A). In the experiments where both PCR and LCR were performed (Fig. 4), only probes 1 and 4 were haptenated. Probes were purified on a 12% denaturing polyacrylamide gel (29). Quantitation was by absorbance at 260 nm.

### LCR and PCR amplification

LCR and PCR reactions contained 500 ng human placental DNA with either no target DNA (negative control) or with 100 molecules of target DNA unless stated otherwise. LCR reactions were run in a buffer containing 50 mM EPPS, pH 7.8, 30 mM $MgCl_2$, 20 mM $K^+$, 10 μM NAD, 1–10 μM gap filling nucleotides, 30 nM each oligonucleotide probe, 1 U *Thermus flavus* DNA polymerase, lacking 3′→5′ exonuclease activity (MBR, Milwaukee, WI), and 5000 U *T.thermophilus* DNA ligase (Abbott Laboratories; 1 U is the amount of DNA ligase producing 1 nM ligated product in 10 min at 55°C at pH 7.8). Reaction volume was 50 μl and each reaction was overlaid with 50 μl mineral oil prior to cycling in a Perkin Elmer 480 thermocycler. Cycling conditions consisted of a 30 s incubation at 85°C and a 30 s incubation at 60°C. Cycle numbers are indicated in figure legends. PCR reactions were run under the same conditions as LCR, except all four dNTPs were used and probes 2 and 3 and ligase were omitted. For the amplification of HIV sequences, concentrations of LCR reagents were as described above except 0.5 U DNA polymerase was used. The reaction cycling conditions consisted of a 3 min denaturation at 94°C followed by 38 cycles of 1 s at 94°C, 1 s at 58°C and 30 s at 64°C.

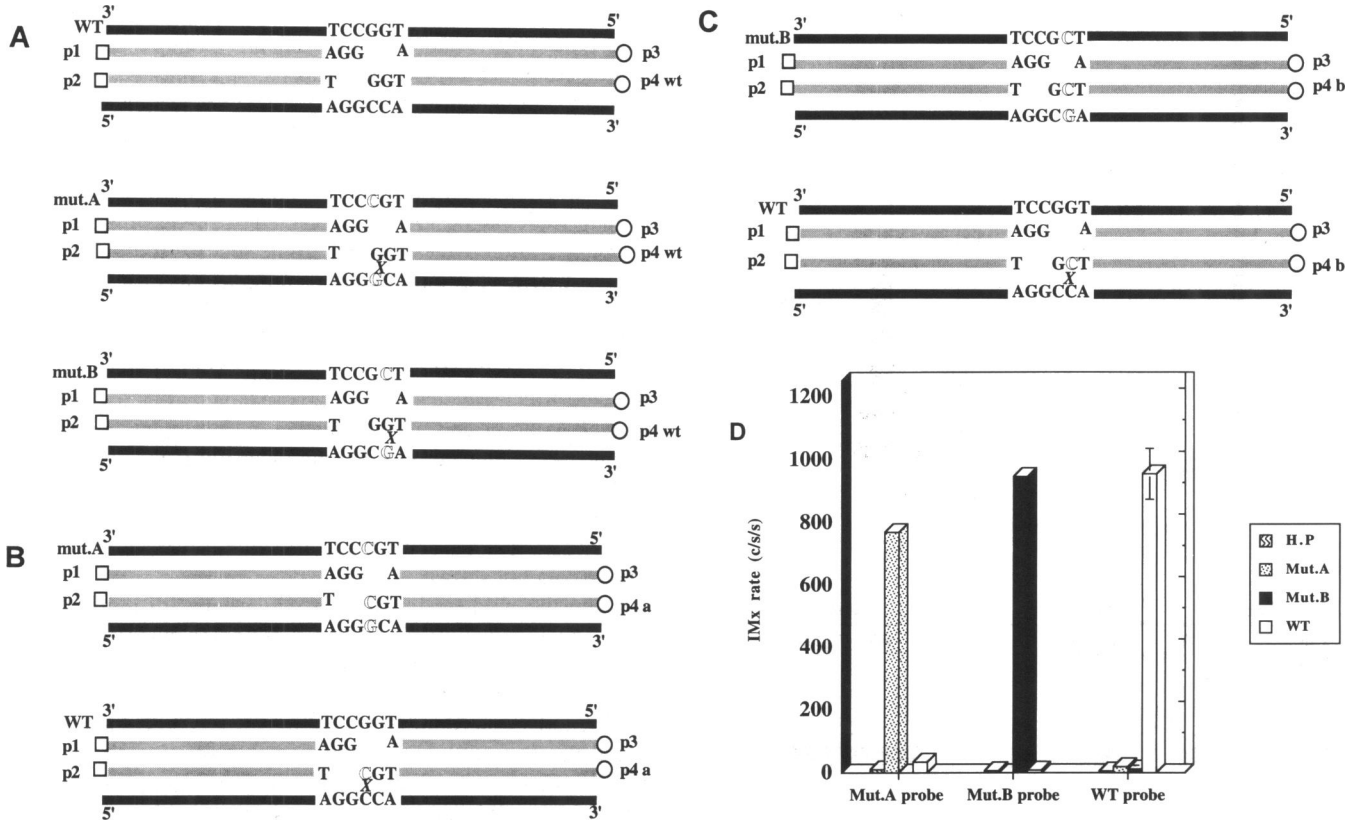


**Figure 2.** Design and specificity of Gap-LCR probe sets for the amplification of DNAs that differ by a single base pair. Double-stranded synthetic target DNAs, designated wild-type (WT), mutant A or mutant B are shown. Only nucleotides of interest are shown; the remainder of the sequences represented by black bars are identical in all targets. The changed nucleotides in the mutant A and mutant B targets with respect to the wild-type target are highlighted. Gap-LCR probes are represented as gray bars. Carbazole is represented as squares and adamantane as circles. (**A**) Gap-LCR probes for the specific amplification of the wild-type target. Probe 4 is complementary to the wild-type target and has a single mismatch (X) with mutant A and mutant B targets. (**B**) Gap-LCR probes for the specific amplification of the mutant A target. Probes 1, 2 and 3 are the same as in (A), probe 4a is different from probe 4wt and the change is highlighted. P4a is complementary to the mutant A target and has a mismatch (X) with the wild-type target. (**C**) Gap-LCR probes for specific amplification of the mutant B target. The change in probe 4b is highlighted. P4b is complementary to the mutant B target and has a mismatch (X) with the wild-type target. (**D**) Specificity of the Gap-LCR probes. the wild-type-, mutant A- and mutant B-specific probe sets shown in (A–C) respectively were tested with either human placental DNA (H.P.), wild type (WT) target, mutant A or mutant B targets. Reaction conditions are described in Materials and Methods. Samples were cycled for 25 cycles and products were detected using the Abbott IMx® automated immunoassay as counts/second/second (c/s/s) as described (17).

### Detection of amplified products

Amplification products were detected via a sandwich immunoassay performed using the Abbott IMx® automated analyzer. Amplification products were captured using anti-carbazole coated microparticles. After a washing step, the captured products were detected using an anti-adamantane–alkaline phosphatase conjugate which, in the presence of methylumbelliferone phosphate, generates a fluorescent product at a rate proportional to the amount of captured product. The average IMx® rate from duplicate samples was taken and standard deviations are shown.

For the detection of LCR products on polyacrylamide gel (Fig. 3B), unhaptenated probe 1 was phosphorylated at the 5′ end using the Gibco BRL 5′ DNA terminus labeling system and 50 µCi [$\gamma$-$^{32}$P]ATP (Amersham). LCR reactions were set up as described above, except that equal amounts of radiolabeled and cold probe 1 were used (15 nM of each per reaction), and samples were cycled for 43 cycles. For restriction analysis, 15 µl of the amplified product was incubated with 1.5 µl *Hae*III (10 U/µl) and 1.8 µl 10 × buffer (Promega) for 1 h at 37°C; the controls (– lanes) were also incubated with 10 × buffer at 37°C in the absence of *Hae*III. Products were separated by electrophoresis on 12% denaturing polyacrylamide gels (29).

## RESULTS

### Design of targets and Gap-LCR probe sets

Synthetic double-stranded DNA targets, designated wild-type or mutant, that differed by a single base pair were designed as shown in Figure 2A. Gap-LCR probe sets specific for the amplification of each target DNA were synthesized. The probes were staggered, i.e. probes 1 and 4 had 3′ overhangs when hybridized to their complements. When annealed to the target DNA, probes 1 and 4 were extended by DNA polymerase, in the presence of appropriate nucleotide(s). The extended probes were then ligated to probes 3 and 2 respectively. The ligated products could function as targets in subsequent cycles, thus allowing exponential amplification. Probes 1, 2 and 3 were common and probe 4 was specific for each target; probes 4wt, 4a and 4b were designed to specifically amplify wild-type, mutant A and mutant B targets respectively and had a single mismatch with the non-analogous

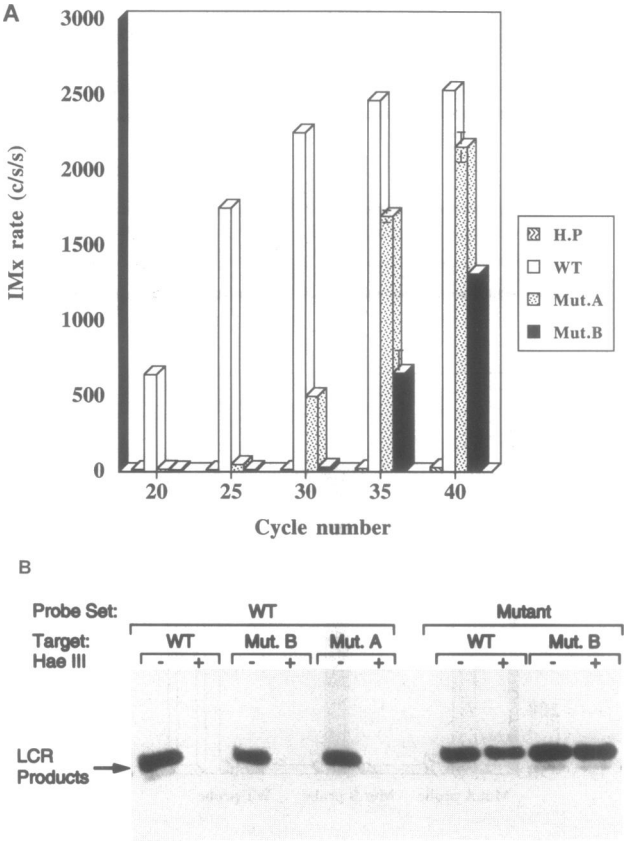


**Figure 3.** (**A**) The effect of cycle number on specificity of Gap-LCR. the wild-type-specific probe set was used with human placental (H.P.), wild-type (WT), mutant A and mutant B DNAs as shown in Figure 2A. Reaction conditions are as described in Materials and Methods and cycle numbers are indicated. (**B**) Analysis of 'overamplified' LCR products. the wild-type-specific probe set was tested with wild-type, mutant B and mutant A targets as shown in Figure 2A. the mutant B-specific probe set was tested with wild-type and mutant B target as shown in Figure 2C. Probe 1 was radiolabeled with $^{32}P$ and reactions were cycled for 43 cycles. Amplified products were divided in two; one set was restricted with *Hae*III (+ lanes), the other set was not (– lanes). Products were electrophoresed on a 12% denaturing gel and detected by autoradiography.

targets (Fig. 2A–C). The mutation was positioned so that on one strand it was complementary to one of the bases to be filled during the extension of probe 1 and on the other strand it was mismatched with respect to probe 4. To assess the effect of the mismatch position on specificity, the position of the mutation was varied to generate a C:C mismatch either at the ultimate 3′ end or penultimate 3′ end of probes 4a and 4b with respect to the wild-type target (Fig. 2B and C). Similarly the wild-type-specific probe, 4wt, had a G:G mismatch either at the ultimate 3′ end or penultimate 3′ end with mutant A and mutant B targets respectively (Fig. 2A).

## Specificity of Gap-LCR probe sets

The specificity of the probe sets is shown in Figure 2D. Mutant A, mutant B, wild-type or human placental DNA (negative control) were amplified with the different probe sets. With human placental DNA, amplified product was not observed, indicating that target-independent non-specific amplification was not significant. Mutant probe sets amplified only their respective mutant targets, whereas the wild-type probe set amplified only the wild-type target and not the mutant targets. These results demonstrate that a single base mismatch positioned either at the ultimate 3′ or penultimate 3′ end of probe 4 is sufficient to provide discriminative amplification by Gap-LCR under the conditions used in this study.

As has been shown for ASPCR and LCR (30), discrimination by Gap-LCR can be adversely affected by increasing the number of amplification cycles. To determine the maximum number of cycles where the amplification remains specific, wild-type, mutant A, mutant B and human placental DNAs were amplified with the wild-type-specific probe set in the presence of dCTP for 20, 25, 30, 35 or 40 cycles (Fig. 3A). Amplified product was detected after 20 cycles with wild-type target, after 30 cycles with mutant A target and after 35 cycles with mutant B target. No product was detected with human placental DNA even after 40 cycles. This result suggests that there is a window of about 10 cycles where the amplification is most specific. Similar results were observed when mutant-specific probes were used with each target (data not shown).

When the wild-type probe set was used with mutant targets (Fig. 2A), identical specificity was seen when dGTP was omitted or added to the reaction, suggesting that omission of the nucleotide to fill the base complementary to the mutation does not significantly contribute to specificity (data not shown). This result was expected, since extension of probe 1 with dCTP and dGTP and ligation to probe 3 would not generate a perfect substrate for probes 2 and 4; probe 4 would still be mismatched with the ligated substrate and be refractory to amplification (Fig. 2A). In contrast, extension from the mismatched probe 4 and ligation to probe 2 would generate a ligated product that would be a perfect substrate for probes 1 and 3, in which case dGTP would not be needed and wild-type product would be generated (Fig. 2A). This prediction was confirmed experimentally by analyzing the products that were generated after over-amplification. wild-type and mutant targets were amplified with the wild-type probe set for 43 cycles (where products from mismatched targets are generated) in the presence of both dGTP and dCTP and the nature of the amplified products was analyzed (Fig. 3B). Amplified products were digested with the restriction enzyme *Hae*III, which cleaves at the GGCC site which would be present only on wild-type products (Fig. 2A). For this experiment, the 5′ end of probe 1 was radiolabeled and the products were detected on a denaturing polyacrylamide gel. The results demonstrate that the products generated from both matched and mismatched targets were cleaved by *Hae*III, thus wild-type product was generated in all cases (Fig. 3B). In contrast, products amplified with the mutant B-specific probe set were not cleaved by *Hae*III. Products generated with the mutant A-specific probe set were not cleaved by *Hae*III either (data not shown). These results confirm the prediction that dGTP is not utilized in the generation of products when the wild-type probe set is used with mutant targets. Therefore omission of dGTP does not significantly contribute to the specificity.

We explored the specificity of Gap-LCR with increasing number of target molecules to determine the maximum number of mismatched target molecules where the amplification remains specific. the wild-type probe set was tested with increasing concentrations of matched (wild-type) or mismatched (mutant) targets (Fig. 4A). The results indicate that while 10 molecules of the matched target were detected, using optimal cycle numbers,

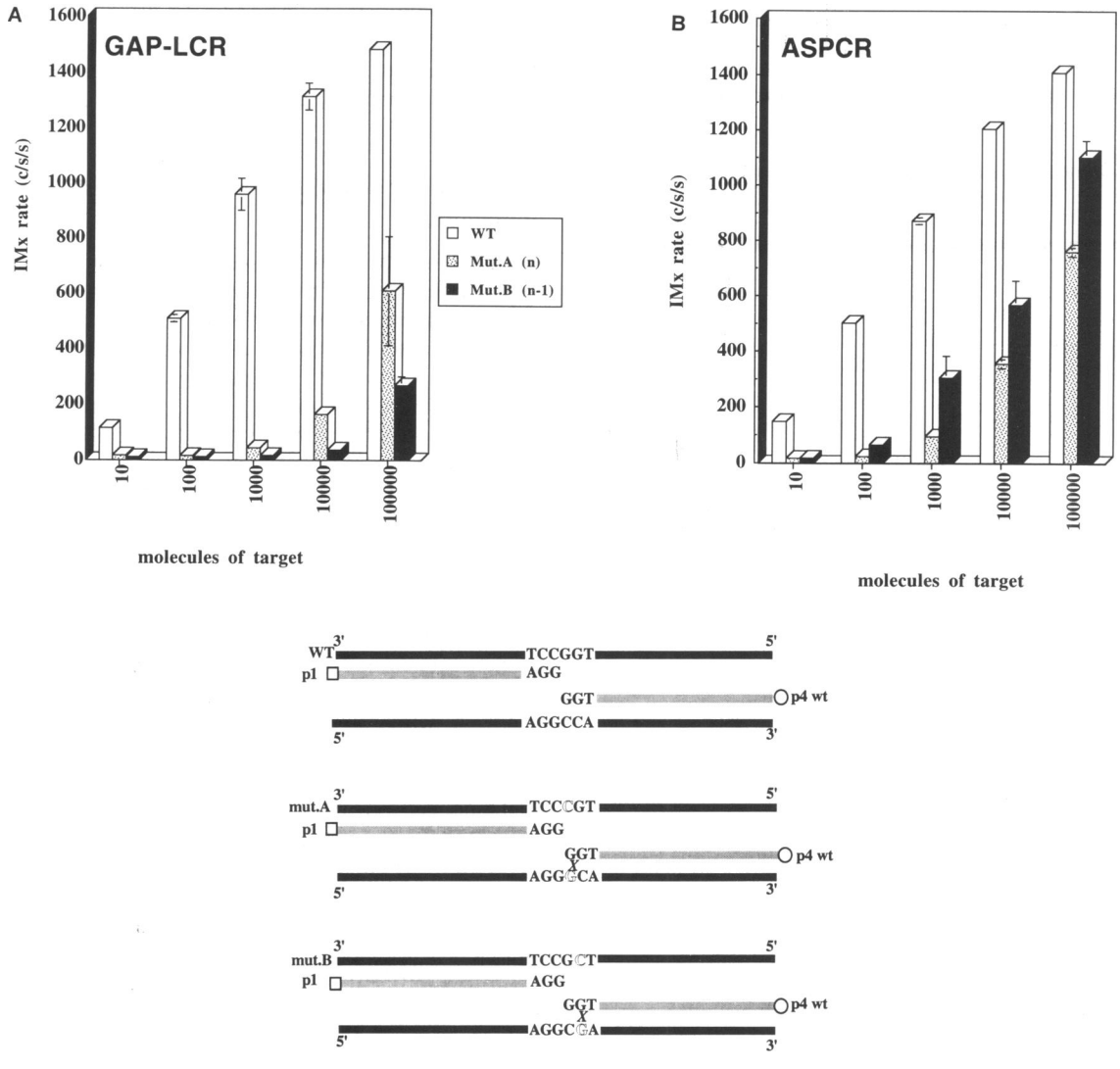


**Figure 4.** Comparison of Gap-LCR to ASPCR. (**A**) The wild-type-specific Gap-LCR probe set was tested with wild-type, mutant A and mutant B targets as shown in Figure 2A, except only probes 1 and 4 were haptenated. Target concentrations are as shown. (**B**) PCR reactions were as described in Materials and Methods; reactions were cycled for 23 cycles. The primers used for PCR are shown in the lower panel. They are identical to probes 1 and 4 used in Gap-LCR (Fig. 2A). The primers are specific for amplification of the wild-type target. Primer 4 has a mismatch at the ultimate 3′ end with the mutant A target and at its penultimate 3′ end with the mutant B target (Xs).

detection of the mismatched targets occured only with $10^4$–$10^5$ molecules. The loss of discrimination with mutant A target ($10^4$ molecules) preceded the loss of discrimination with mutant B target ($10^5$ molecules). Similar results were seen when the cycle number was increased beyond the optimum (Fig. 3A); product was detected after 30 cycles with mutant A target and 35 cycles with mutant B target. Both mutant targets amplified at the same rate with their respective matched probes and the differential rate of amplification of mutant targets was only observed with mismatched wild-type probes. These results suggest that a mismatch positioned at the penultimate 3′ end is discriminated better than a mismatch at the ultimate 3′ end.

In Gap-LCR, discrimination between targets that differ by a single base may rely on three steps: (i) hybridization of mismatched probes; (ii) fidelity of the polymerase to extend from mismatches; (iii) specificity of the ligase to join probes extended from mismatches. ASPCR also requires the first two steps, yet Gap-LCR may have the additional level of specificity required by the necessity for proper ligation. To address this question, specificity of Gap-LCR and ASPCR were compared under the same reaction conditions (Fig. 4). ASPCR experiments were performed with the same targets using only two of the haptenated probes (probes 1 and 4), all four nucleotides and same reaction conditions utilized for Gap-LCR. For this comparative study, only probes 1 and 4 were linked to haptens for Gap-LCR. Detection of products relied on complementarity of the strands linked to the two haptens. Results indicate that with ASPCR, better discrimination was observed when the mismatch was at the ultimate 3′ end than at the penultimate 3′ end. This is in contrast to the observation made using Gap-LCR (Figs 3A and 4A). Comparing the two amplification procedures, mismatched targets were amplified at a faster rate in ASPCR than Gap-LCR, while the amplification rate of the matched target was equivalent in both reactions. This difference was enhanced when the mismatch was

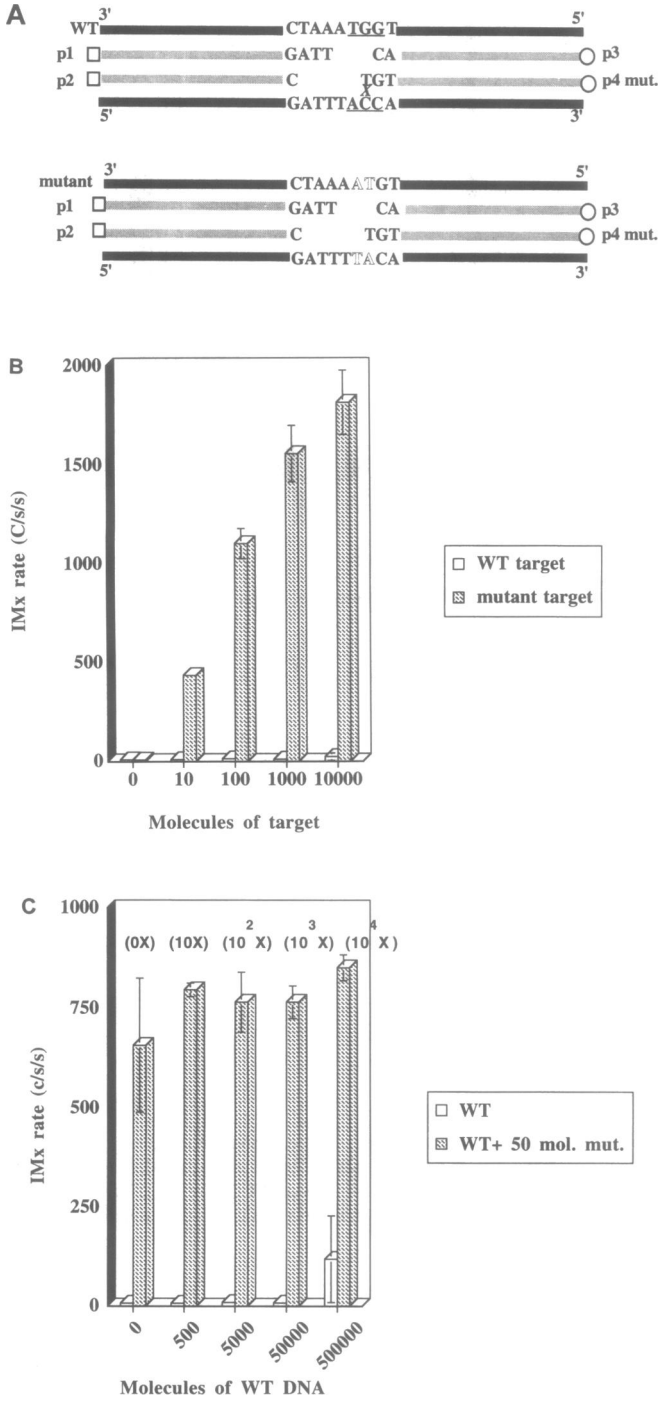


**Figure 5.** Gap-LCR for the detection of an AZT resistance mutation. (**A**) Design of the Gap-LCR probe set for specific amplification of the mutant DNA. The wild-type and mutant target DNAs comprising 50 bases of the HIV reverse transcriptase gene are shown as solid bars. Codon 215 is underlined. The mutations in codon 215 are highlighted. Probes are represented as gray bars and haptenated as described in Figure 2. Probe 4mut. is specific for amplification of the mutant DNA and has a 3′ terminal mismatch (X) with the wild-type target. (**B**) The mutant-specific probe set was tested with increasing concentrations of wild-type or mutant targets as shown in (A). Reaction conditions are as described in Materials and Methods. (**C**) The mutant-specific probe set was tested with 50 molecules of mutant target mixed with increasing concentrations of the wild-type target (shaded bars) or with increasing concentrations of the wild-type target alone (open bars). The ratio of the wild-type target to the mutant target is indicated above the shaded bars. Reaction conditions are as described in Materials and Methods.

at the penultimate 3′ end. This observation suggests that the specificity of Gap-LCR does not solely rely on hybridization and the extension of the mismatched probe by the polymerase as in ASPCR and that the ligation step adds to the specificity of Gap-LCR.

### Gap-LCR for detection of a HIV AZT resistance mutation

To show the feasibility and specificity of Gap-LCR for detection and discrimination of a natural mutation, a mutation at codon 215 of the HIV reverse transcriptase gene was tested as a model system. Mutation at codon 215 from ACC (threonine) to TAC (tyrosine) has been associated with resistance to AZT (3′-azido-3′-deoxythymidine) (31). Probe sets specific for the amplification of the mutant viral DNA were designed and tested on cloned HIV DNA carrying wild-type or mutated sequences at codon 215 (Fig. 5A). Even though the wild-type and mutant targets differ by two bases, one of them (the first base of the codon) is positioned in the overlapping gap and does not create a mismatch with the probes. Because the necessary nucleotides are provided in the reaction (ATP and TTP), the change in that position does not contribute to discrimination. Thus, the discrimination relies on a single base change; the mutant-specific probe set is designed to have a single mismatch at the ultimate 3′ end of probe 4 with the wild-type DNA. The specificity of the mutant-specific probe set was tested with increasing concentrations of wild-type and mutant targets. While 10 molecules of mutant target were detected, no product was observed with up to 10 000 molecules of wild-type target (Fig. 5B). No product was detected with human placental DNA (used as a negative control). To determine whether the mutant target could be detected in the presence of excess wild-type target, 50 molecules of mutant target were mixed with increasing concentrations of wild-type target ($5 \times 10^2$ to $5 \times 10^5$). Increasing concentrations of wild-type target in the absence of mutant target was tested as the control. Results indicate that specific amplification of the mutant target occurs in the presence of up to $10^4$-fold excess wild-type target (Fig. 5C). Slight cross-reactivity with the wild-type target was observed when $5 \times 10^5$ molecules of wild-type target were used.

## DISCUSSION

A reliable DNA diagnostic method requires accurate discrimination, low background and automation. In this study we have shown that Gap-LCR meets these requirements; DNA targets that differ by a single base pair were discriminated, the background was low, sensitivity was high and the products of the reaction were detected by an automated immunoassay. In the experiments designed in this study, discrimination between related targets relied on a single base mismatch between one of the Gap-LCR probes and the target DNA. The extent of the discrimination depended on the cycle number, concentration of the mismatched target and the position of the mismatch.

As with any other amplification reaction, the reaction specificity was expected to deteriorate with increasing cycle number (30). When mismatched probes are extended and ligated during any cycle, the newly formed molecules are able to function as templates in subsequent cycles. The products generated from the matched target will reach a plateau after a certain cycle number, while the products generated from the mismatched target will continue to exponentially amplify until they reach the levels seen

with the matched target, at which point discrimination will be completely lost. A rough calculation of the accumulation of products from matched and mismatched targets has been reported previously for ASPCR by Ugozolli and Wallace (30). We show that for Gap-LCR accumulation of products from mismatched targets occurs about 10–15 cycles later than the detection of products from the matched target. Moreover, even after 20 additional cycles (40 cycles total), no signal was observed with negative control placental DNA.

Template concentration also plays a major role in specificity. Similarly to an increased number of cycles, the specificity of Gap-LCR was expected to deteriorate when large amounts of mismatched target were used. We showed that under the conditions used in this study, as few as 10 molecules of the matched target were detected, while equivalent detection of the synthetic mismatched target required 10 000–100 000 molecules, depending on the position of the mismatch. With the HIV-specific probe set, the specificity was even better. Product was detected with 10 molecules of the matched target, while only a small amount of product was detected with 500 000 molecules of the mismatched target. The better specificity observed with the HIV probe set may be attributed to differences in sequence, in reaction conditions and/or the nature of the targets used in these studies (50 bp linear double-stranded synthetic targets versus 8 kb circular plasmid DNA). Our results indicate that with Gap-LCR, good specificity can be obtained under conditions where exquisite sensitivity is maintained.

Our studies demonstrate that a single mismatch between one of the Gap-LCR probes and the target is sufficient for discrimination of single base substitutions. The specificity seems to rely solely on the efficiency of extension and ligation of the mismatched probe. Omission of the nucleotide complementary to the mutated base in the fill does not significantly add to the specificity. Once the mismatched probe is extended and ligated, it generates a target for the complementary probes, which can extend and ligate in the absence of the omitted nucleotide. After such an event, amplification is exponential in the following cycles. The omission of the nucleotide complementary to the mutation would effectively prevent amplification if the mutation was positioned in an overlapping gap, where probes need to be designed not to cover the mutated base in either strand. However, such a scheme has limited application. It can only be used if the mutation is an A or T change to a C or G or vice versa. Other changes would necessitate the same fill in the overlapping gap.

In several previous reports where single base mismatches were not refractory to amplification, further deliberate mismatches were introduced to achieve discrimination with ASPCR or blunt-end LCR (10,11,30,32–35). For ASPCR, Newton *et al.* (10) reported that the primers became increasingly refractory to amplification as the additional mismatch was moved progressively closer to the 3′ end of the PCR primer. Under the conditions used in our study, a second mismatch was not necessary and in fact positioning a second mismatch next to the terminal mismatch would likely result in a failure to amplify either target, since we demonstrated that a single base mismatch one base from the 3′ end was inhibitory to amplification. We have also observed that a mismatch two bases from the 3′ end was refractory to amplification with Gap-LCR (data not shown). Reaction conditions may be optimized to accommodate additional mismatches. Whether such an approach would increase the specificity of Gap-LCR remains to be explored.

It was previously reported that the nature of the mismatch affects both the polymerase extension and ligation efficiencies (10,14,32,33). However, in ASPCR conflicting results were obtained for the same mismatches in different reports, presumably due to differences in primer length, surrounding sequences and reaction conditions (30). Although our studies were not designed to compare the effect of base pair composition on the specificity of Gap-LCR, we observed that G:G, C:C and C:T mismatches were all refractory to amplification with Gap-LCR. Nevertheless, to assess the effect of mismatch position on Gap-LCR specificity, we chose to limit our comparison to the same mismatch (G:G), positioned at two different locations at the ultimate or penultimate 3′ end of probe 4wt. We found that better specificity was obtained when the mismatch was at the penultimate 3′ end. Previous studies on the effect of mismatch positioning on the specificity of polymerases or ligases are limited in scope. The effect of mismatches for *Taq* polymerase and T4 ligase have been shown to be greatest at the ultimate 3′ position (32,36). Our PCR results with *T.flavus* polymerase are in agreement with these observations. Yet in Gap-LCR we observed better specificity with the 3′ penultimate mismatch. This difference may be due to the nature of the ligase used in our studies and/or to the combinatory effect of ligase and polymerase as it is in Gap-LCR. Direct comparison of ligase specificity in Gap-LCR to ligase specificity in the absence of polymerase in blunt-LCR is not feasible. Generation of target-independent ligation products is very common in blunt-LCR and would preceed detection of products from mismatched targets. Moreover, the position of the mismatch in blunt-LCR probe and Gap-LCR probe cannot be directly compared; in Gap-LCR, depending on the size of the gap, a mismatch at the 3′ end of the probe becomes a mismatch 2–4 bases away from the ligation junction after polymerase extension.

To determine whether discrimination obtained with Gap-LCR was solely due to the specificity of the polymerase or to the additive specificity of polymerase and ligase, we compared discrimination obtained with polymerase alone (in ASPCR) to discrimination obtained with polymerase plus ligase (in Gap-LCR) under the same reaction conditions. Our data indicate that the specificity of Gap-LCR depends on the fidelity of polymerase extension as well as on the specificity of ligation. The difference between Gap-LCR and ASPCR was further enhanced when the mismatch was positioned at the penultimate 3′ base, since at that position the specificity increased for Gap-LCR but decreased for ASPCR when compared to the mismatch at the 3′ end. Specificity of PCR could have been improved by optimizing conditions. However, these experiments were not aimed at comparing the performance of Gap-LCR versus ASPCR; they were designed to determine whether the specificity of Gap-LCR relied on polymerase alone or on polymerase plus ligase, thus the same reaction conditions needed to be utilized.

The potential of this amplification method to detect a mutated DNA sequence present at low copy number in a high background of wild-type DNA was evaluated. We observed that detection of mutant DNA in the presence of up to $10^4$-fold excess wild-type DNA was feasible. This result demonstrates the advantage of Gap-LCR over blunt-LCR. With blunt-LCR, the signal obtained from mutant DNA in the presence of 100-fold excess wild-type DNA could not be distinguished from the background noise (37). Our results suggest that Gap-LCR would allow detection of mutations present at frequencies as low as one in $10^4$ gene copies;

thus Gap-LCR can be used in conditions where only a small fraction of cells are expected to contain the mutation.

In conclusion, we have demonstrated that Gap-LCR is a sensitive and specific amplification technique that can accurately discriminate single base changes. A significant advantage of the Gap-LCR assay described here is the ability to specifically detect the reaction products using a simple automated immunoassay system. Our study suggests that Gap-LCR can be used as a powerful tool in the diagnosis of genetic diseases, in monitoring drug resistant pathogens and in the detection of oncogenic mutations.

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