Ligase chain reaction (LCR)--overview and applications.

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Ligase Chain Reaction (LCR)—Overview and Applications

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PCR has facilitated the development of a variety of nucleic acid-based detection systems for genetic disorders as well as for bacterial, viral, and other pathogens.[1] In the last few years, a number of other DNA amplification methods, including self-sustained sequence replication (3SR),[2] Q-beta replicase (Qβ),[3] and the ligase chain reaction (LCR),[4,5] have been developed to complement, or as alternatives to, PCR.[6,7] From its initial detailed reports in 1991, LCR evolved as a very promising diagnostic technique that is often utilized in conjunction with a primary PCR amplification. LCR employs a thermostable ligase and allows the discrimination of DNA sequences differing in only a single base pair (see Fig. 1).[4,5] The power of LCR is its compatibility with other replication-based amplification methods. By combining LCR with a primary amplification, one effectively lines up the crosshairs to distinguish single base-pair changes with pinpoint accuracy.

The intellectual genesis of LCR can be traced back to pioneering work by Whiteley et al.[8] who described an oligonucleotide probe-based assay using two probes that are ligated together only when immediately adjacent to each other. The same concept is applied in the oligonucleotide ligation assay (OLA).[9,10] This method was used in conjunction with a primary PCR step to screen for sickle cell anemia, the ΔF508 mutation in cystic fibrosis, and T-cell-receptor polymorphisms. Wu and Wallace[11] described a similar technique called the ligase amplification reaction (LAR), which employs two sets of complementary primers and repeated cycles of denaturation (at 100°C) and ligation (at 30°C) using the mesophilic T4 DNA ligase. Use of mesophilic, that is, T4 or *Escherichia coli*, ligase has the drawback of requiring the addition of fresh ligase after each denaturation step, as well as appearance of target-independent ligation products.[11,12] In contrast, LCR provides a much higher sensitivity and is less susceptible to the formation of false-positive ligation products.

Thermostable ligase minimizes target-independent ligation because the reaction can be performed at or near the melting temperature (T_m) of the oligonucleotides.[5] Furthermore, the use of thermostable ligase avoids the need to add fresh ligase after each denaturation step as required in LAR. Recently, thermostable ligase has become available from a variety of commercial suppliers, and this will probably lead to even wider application and use of this new amplification technique.

The concept of LCR and ligation-based diagnostics has been reviewed.[5,13] We will provide an overview of the recent advancements, new developments, and applications of LCR and similar ligase-mediated detection methods.

THEORY OF LCR AND SIMILAR AMPLIFICATION METHODS

The principle of LCR is based in part on the ligation of two adjacent synthetic oligonucleotide primers, which uniquely hybridize to one strand of the target DNA (see Fig. 1). The junction of the two primers is usually positioned so that the nucleotide at the 3′ end of the upstream primer coincides with a potential single base-pair difference in the targeted sequence. This single base-pair difference may define two different alleles, species, or other polymorphisms correlated to a given phenotype. If the target nucleotide at that site complements the nucleotide at the 3′ end of the upstream primer, the two adjoining primers can be covalently joined by the ligase. The unique feature of LCR is a second pair of primers, almost entirely complementary to the first pair, that are designed with the nucleotide at the 3′ end of the upstream primer denoting the sequence difference. In a cycling reaction, using a thermostable DNA ligase, both ligated products can then serve as templates for the next reaction cycle, leading to an exponential amplification process analogous to PCR amplification. If there is a mismatch at the primer junction, it will be discrimi-

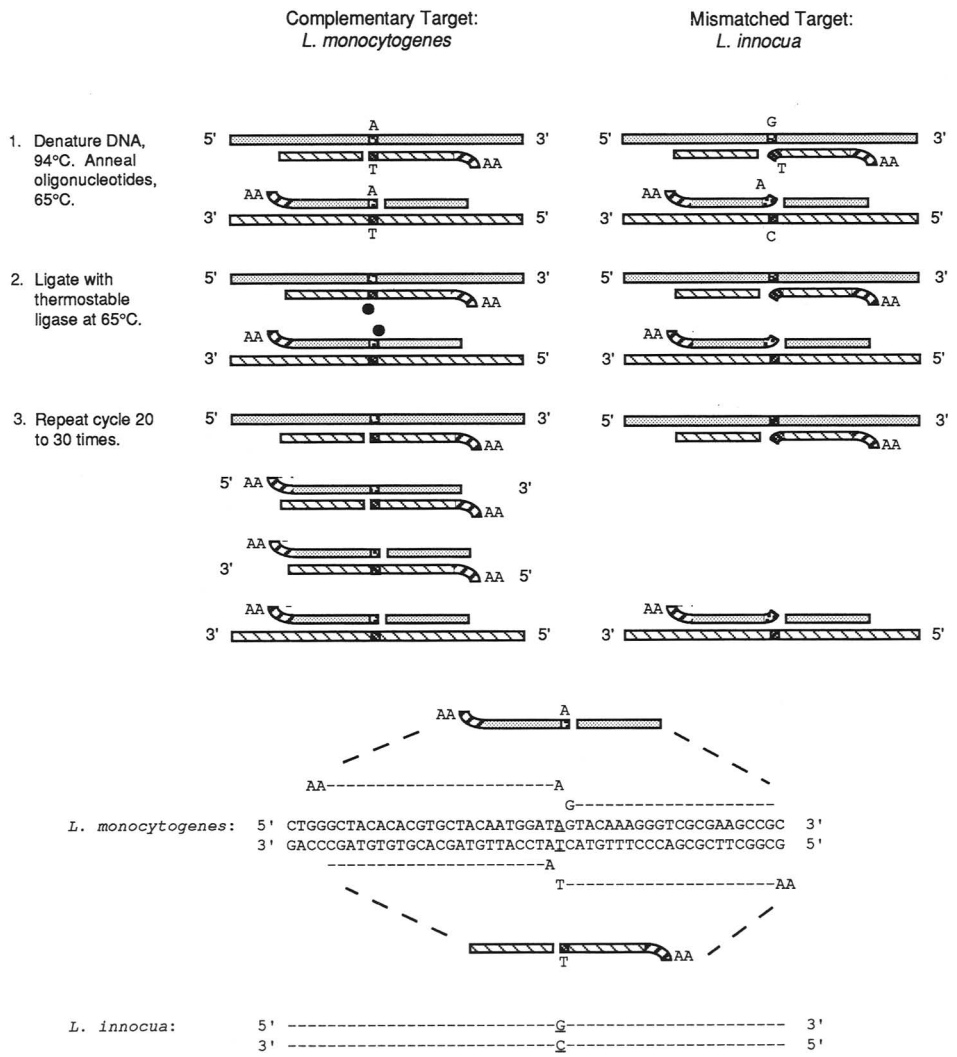

FIGURE 1 Principle of LCR. (*Bottom*) The example shown is an LCR with matched target (*L. monocytogenes*) and mismatched target (*L. innocua*). The pathogenic bacteria *L. monocytogenes* can be distinguished from other closely related *Listeria* spp. (e.g., *L. innocua*) by a single base-pair difference in the 16S rDNA.[20] *L. monocytogenes* has an A-T base pair at nucleotide 1258, whereas *L. innocua* has a G-C base pair at this position. (*Top*) DNA is denatured at 94°C, and the four LCR primers anneal to their complementary strands at 65°C, which is approximately 5°C below their T_m. Thermostable ligase (●) will only ligate primers that are perfectly complementary to their target sequence and hybridize directly adjacent to each other (as shown with *L. monocytogenes, left*). The discriminating bases at the 3′ ends of the upstream primers are depicted as boxes on the target as well as on the primers for clarity. Primers that have at least a single base-pair mismatch at the 3′ end contributing to the junction of the two primers will not ligate (as shown with *L. innocua, right*). The discriminating primers have a 2-bp noncomplementary AA tail at their 5′ ends to avoid ligation of the 3′ ends.

nated against by thermostable ligase and the primers will not be ligated. The absence of the ligated product therefore indicates at least a single base-pair change in the target sequence.[4] Ligase detection reaction (LDR) is similar to LCR.[5] In LDR, one pair of adjacent primers that hybridize to only one of the target strands is used to achieve a linear amplification (see Fig. 2). LDR may be used following a primary amplification (PCR, 3SR, Qβ-replicase, RT–PCR) and has the advantage of accurately quantitating the ratio of two alleles in a target sample.[14] LDR coupled to PCR has promise in a multiplex format where several mutations are analyzed in a single amplification.[5] This

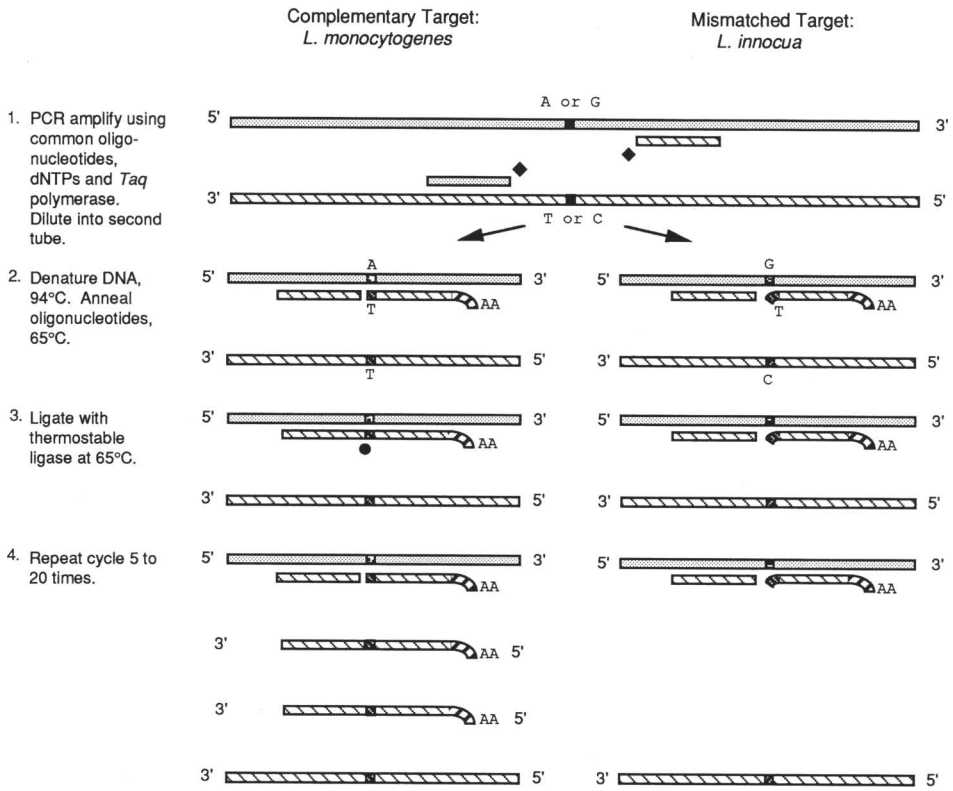

FIGURE 2 Principle of PCR-coupled LDR. The same two target sequences as in Fig. 1 are used to illustrate the PCR-coupled LDR. The DNA stretch containing the single base-pair difference that distinguishes *L. monocytogenes* from *L. innocua* (see Fig. 1, *bottom*) is PCR amplified using PCR primers outside the region of the LCR primers. The PCR amplifies both target sequences under standard conditions (details can be found in Refs. 20 and 32). (♦) *Taq* polymerase 3′ to each of the two PCR primers. After PCR amplification, *Taq* polymerase is inactivated by 97°C for 25 min.[33]. An aliquot of the PCR-amplified DNA (between 1% and 4% of the PCR reaction) is then used in the LDR. DNA is denatured at 94°C, and the two LDR primers anneal to their complementary strand at 65°C, which is approximately 5°C below their T_m. As in LCR (Fig. 1), the thermostable ligase (●) will only ligate primers that are perfectly complementary to their target sequence and hybridize directly adjacent to each other (as shown with *L. monocytogenes, left*). Primers that have at least a single base-pair mismatch at the 3′ end contributing to the junction of the two primers will not ligate (as shown with *L. innocua, right*). An LDR cycle, which consists of a denaturing step at 94°C for 1 min and an annealing step of 65°C, is repeated 5–20 times so that a linear amplification of ligated LDR primers is achieved with the complementary target (i.e., *L. monocytogenes*).

method is currently being applied to the simultaneous detection of multiple mutations in cystic fibrosis[15,16] as well as in 21-hydroxylase deficiency.[17]

pLCR is another ligase-mediated detection method, where the 3′ ends of the discriminating (or allele-specific) primers coincide with a potential base-pair change. The pLCR primers are designed with a gap between the discriminating and the nondiscriminating primer (see Fig. 3). In this reaction, the gap is filled using the *Taq* polymerase Stoffel fragment, followed by the ligation of the elongated discriminating primer with the nondiscriminating primer. The specificity of this method relies on allele-specific elongation of the discriminating primer by the polymerase. Birkenmeyer and Mushahwar[18] described another ligase-mediated technique called gapped LCR (G-LCR). This technique uses four oligonucleotide primers with the two primers, of each pair being separated by a gap of one or more consecutive bases that are specific for the target DNA (see Fig. 4). By adding only the missing deoxynucleotides to the reaction together with a thermostable polymerase and a

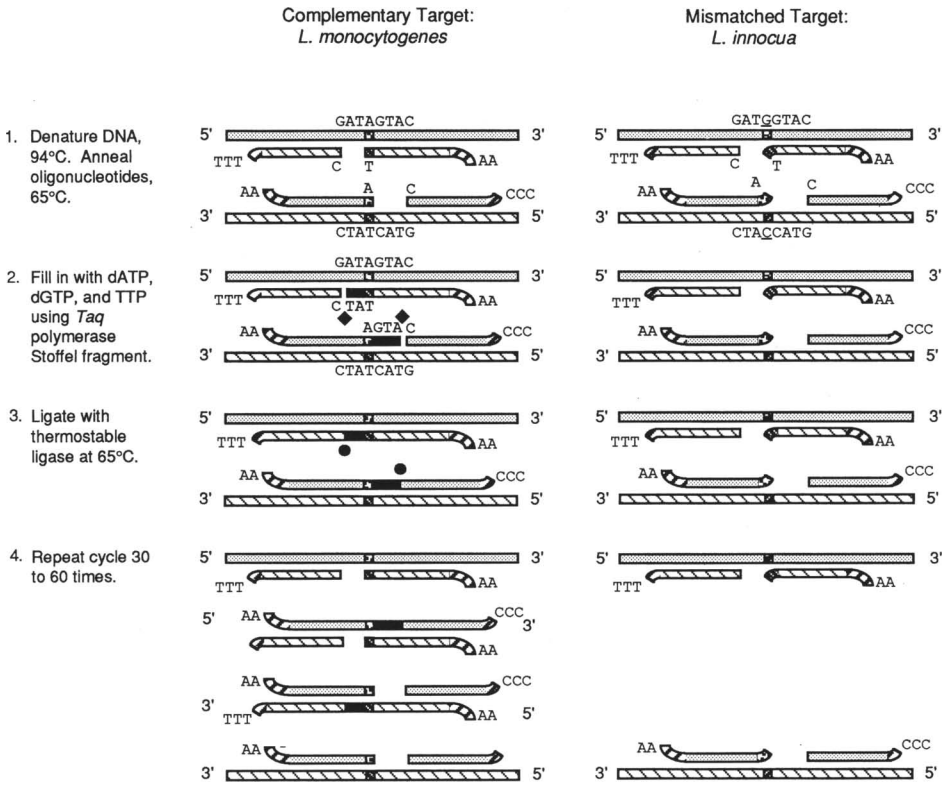

FIGURE 3 Principle of pLCR. The same target sequences as in Fig. 1 are used to illustrate pLCR. After the denaturing of the DNA at 94°C, the four pLCR primers are allowed to anneal at 65°C. These primers anneal so that a two- or three-nucleotide gap between the primers of one pair (which anneals to the same strand) is formed. The 3′ end of the discriminating primers (shown with a shaded box at the 3′ end and with the discriminating nucleotides indicated by T and A) can be elongated by the *Taq* polymerase Stoffel fragment and the appropriate nucleotides analogous to the process in PCR. Only the nucleotides needed to fill the two- or three-nucleotide gap between the discriminating and the nondiscriminating primers are included in the reaction mix; dATP, dGTP, and dTTP are needed for the example shown. After elongation of the discriminating primers by two or three nucleotides, the junction between the elongated discriminating primer and the nondiscriminating primer can be sealed by the thermostable ligase. This cycle is repeated between 30 and 60 times. With a noncomplementary target (*right*), no elongation of the discriminating primer is possible; therefore, no ligation of the two primers will occur and no pLCR product will form.

thermostable ligase, the gap must first be filled in the presence of the matching target and before the resulting nick can then be sealed by the ligase. This technique limits itself to the detection of base-pair changes from A-T/T-A to G-C/C-G or vice versa. For example, gapped LCR could not distinguish β^A globin from β^B globin (A → T transversion) because there is no difference in the bases required for filling the gap. A similar principle is also applied in the repair chain reaction (RCR), which has been used for the detection of human papillomavirus (HPV) 16.[19]

COMPARISON OF LCR, pLCR, AND G-LCR

One performance-linked difference cited among LCR, pLCR, and G-LCR is the relative amount of ligated product in the absence of template. Because both pLCR and G-LCR require an initial template-dependent extension, they have been proposed to be less prone to false positives in the absence of template. To compare LCR, pLCR, and G-LCR, the appropriate primers for the detection of *Listeria monocytogenes* by these three techniques were designed and synthesized.[20,21] Locations of the primers are shown in Figures 1, 3, and 4.

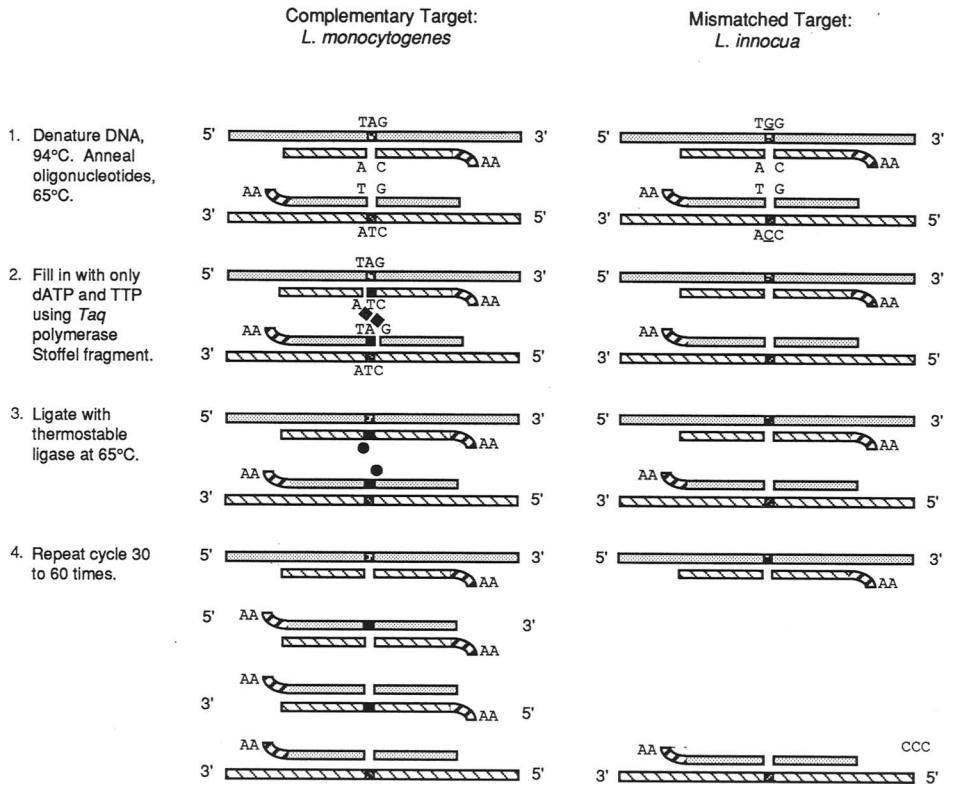

FIGURE 4 Principle of G-LCR. The same target sequences as in Fig. 1 are used to illustrate G-LCR. After denaturing of the DNA at 94°C, the four primers are allowed to anneal at 65°C. These primers anneal so that a one-nucleotide gap between the primers of one pair (which anneals to the same strand) is formed. This gap is located so that it coincides with the base pair discriminating the two targets (*L. monocytogenes* and *L. innocua* in the example shown) from each other. The 3′ end of the downstream primer can be elongated by the *Taq* polymerase Stoffel fragment and the appropriate nucleotides analogous to the process in PCR. Only the nucleotides needed to fill the one-nucleotide gap (shown as a shaded box in the target sequence with the discriminating nucleotides indicated by T and A) between the two primers are included in the reaction mix; only dATP and dTTP are needed for the example shown. After elongation of the discriminating primers with the appropriate nucleotide, the junction between the elongated downstream primer and the upstream primer can be sealed by the thermostable ligase. This cycle is repeated between 30 and 60 times. With a noncomplementary target (*right*), no elongation of the discriminating primer is possible; therefore, no ligation of the two primers will occur and no G-LCR product will form.

Radioactively labeled LCR primers and detection of the ligation products after gel electrophoresis (for details, see "Detection Methods of LCR products") were used to compare the three methods for their ability to detect single base-pair differences in PCR-amplified 16S rDNA. The reaction conditions for G-LCR as well for pLCR are as described in Table 1 for pLCR. In contrast to previous reports for G-LCR, the *Taq* polymerase Stoffel fragment was used instead of *Taq* polymerase. Compared with *Taq* polymerase, the Stoffel fragment lacks 5′ → 3′ exonuclease activity and does not excise bases from the 5′ end of the primer adjacent to the gap. No target-independent ligation products were observed for LCR, pLCR, or G-LCR. Furthermore, a clear differentiation of *L. monocytogenes* from *L. innocua* based on a single base-pair difference in the 16S rDNA was possible for all three formats. This is the first time that a single base-pair difference was detected using G-LCR. Previous reports described the discrimination of targets with at least two base-pair differences.[22,23] Furthermore, this shows that LCR, pLCR, and G-LCR have the potential to detect single base-pair differences; their discriminatory ability

TABLE 1 Protocols for LCR, pLCR, and G-LCR

	LCR	pLCR	G-LCR
Detailed references	4, 5, 20, 27, 30, 32, 41, 42	21	22, 23
Position of discriminating nucleotide	3′ base of both strands (single base 3′ overhang)	3′ base of both strands (1 or 2 bases overhang)	nucleotides to be filled in
T_m of primers	66–70°C[4,20] or 60–66°C.[42]	68–70°C	62–76°C
Amount of each primer	1–10 fmoles/μl	2 fmoles/μl	16.6–20 fmoles/μl
Labeling of primers	biotin/digoxigenin; fluorescein; ^{32}P	^{32}P	biotin/fluorescein; unlabeled, used with ^{32}P-labeled nucleotides for fill-in reaction
Reaction volume	10–50 μl	25 μl	25–50 μl
Buffer conditions	20–50 mM Tris-HCl (pH 7.6), 100 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM DTT, 1 mM NAD^+, 0.1–0.01% Triton X-100[a]	80 mM KOH/KCl, 50 mM EPPS, 10 mM $MgCl_2$, 10 mM NH_4Cl, 1 mM DTT, 10 μg/ml BSA, 1 mM NAD^+	80 mM KOH/KCl, 50 mM EPPS, 10 mM $MgCl_2$, 10 mM NH_4Cl, 1 mM DTT, 10 μg/ml BSA, 0.1 mM NAD^+
Amount of nucleotides for fill-in reaction	–	1 μM	1 μM
Carrier DNA to suppress background	0.4 μg salmon sperm DNA/μl	–	–
Thermostable enzymes/reaction volume	1.5 nick closing units *Taq* ligase/μl[4,20] or 0.15 U/μl[24]	1.5 nick closing units *Taq* ligase/μl and 0.08 units *Taq* polymerase Stoffel fragment/μl[21]	68 U/μl *Taq* ligase and 0.02 U/μl *Taq* polymerase[22,23]
Cycle conditions	94°C for 1 min, 65°C for 4 min, 10–30 cycles[b] or 94°C for 1 min, 60°C for 8 min; 30 cycles[c]	97°C for 3 min, 1 cycle; 94°C for 1 min, 65°C for 4 min; 50 cycles	heat in boiling water bath for 3 min; then 85°C for 30 sec, 50–60°C for 20 sec–1 min; 27–60 cycles

[a]Barany[4] did not include Triton X-100.
[b]For primers with T_m of 66–72°C.[4,20]
[c]For primers with T_m of 60–66°C.[42]

might nevertheless depend on the nature and composition of the targets. The sensitivity of these three techniques in a comparative study is currently under investigation in our laboratories.

LCR REACTIONS—IMPORTANT FACTORS

Accurate results from LCR assays depend on a variety of factors, including primer design and reaction conditions. Based on our experience and those of others over the past 3 years, a few of the most important factors that need to be considered in the development of LCR assays follow.

Design of LCR Primers

To minimize target-independent ligation, LCR primers with a single base-pair overhang, rather than blunt ends, should be used. The importance of single base-pair overhangs is shown by Kälin et al.,[24] who reported a relatively high amount of target-independent ligation using primers with blunt ends. The T_m

of all four primers of one set of LCR primers should be within a narrow temperature range, ideally with an absolute T_m of 70°C ± 2°C. Furthermore, the primers should be designed so that one primer cannot serve as a bridging template for other primers and therefore lead to target-independent ligation. Adding noncomplementary tails of two nucleotides or longer to the nonadjacent 5′ ends of the primers should prevent ligation of the 3′ ends. Depending on the discriminated nucleotides, different amounts of ligation product are observed with a mismatched target.[4] Expected amounts of false ligation for specific mismatches are shown in Table 2. These data can be used for designing primers with the lowest possible rate of false ligation when some choice between different target sequences exists.

The nature of the base pair at the 3′ end of the primer with the matched target seems to influence the ligation efficiency. Two sets of LCR primers with the corresponding difference at the 3′ end of the discriminating primer were used for the detection of a single base-pair difference (D128G) in the two alleles of the bovine CD18 gene.[25] The discriminating primer set that carries a G on one and a C on the other 3′ end gave a more efficient ligation as compared with the second set of primers in which the discriminating primers carry an A and a T at their 3′ ends. The greater hydrogen bonding of the G-C base-pairing facilitates a more stable hybrid as compared with A-T base-pairing, therefore allowing a more efficient ligation.

LCR Conditions

Standard conditions for a 50-µl LCR are as follows: One set of four primers (between 25 and 200 fmoles of each primer) is incubated in the presence of target DNA in the reaction buffer (50 mM Tris-HCl at pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol, 1 mM NAD^+, 20 µg of salmon sperm DNA) with 75 nick-closing units of *Thermus aquaticus* DNA ligase.[26] The inclusion of 0.01%–0.1% Triton X-100 in the reaction buffer gives a higher ligation rate but also leads to a slight increase of ligation with a mismatched target.[20,27] Reaction cycles are usually 15 sec to 1 min at 94°C for denaturation, followed by 4 min to 6 min at 60–65°C (ideally 5°C below the lowest T_m of the primers). Unlike PCR, there is no extension step between annealing and denaturation. In LCR, this cycling pattern is repeated between 10 and 30 times, but the number of cycles has to be optimized for each assay. In G-LCR, between 30 and 60 cycles with denaturation at 85°C and annealing at 50–53°C have been used.[22,23] Protocols for LCR, pLCR, and G-LCR are outlined in Table 1.

A NAD-requiring thermostable ligase[26,28] is most often used in ligase-based amplification methods. Recently, another thermostable ligase, which requires ATP as a cofactor, has been cloned and sequenced.[29] However, the use of this enzyme in DNA amplification methods has not yet been explored.

DETECTION METHODS FOR LCR PRODUCTS

Detection of the LCR product, that is, the two ligated primers, was initially

TABLE 2 Noise-to-signal ratio for certain mismatches in the LCR

Oligonucleotide base-target base	Noise-to-signal ratio[a] (%)
A-A, T-T	1.1
T-T, A-A	<0.2
G-T, C-A	1.3
G-A, C-T	<0.2

[a]Calculated as amount of product with mismatched primers divided by the amount of product with complementary primers (adapted from Barany[4]).

accomplished by using a ^{32}P radioactive label on the 3′ end of the upstream primer. The separation of LCR products and primers was achieved by denaturing gel electrophoresis, and the LCR product was detected by autoradiography. The level of sensitivity reached in an LCR with this detection method is on the order of 200 target DNA molecules.[4] Winn-Deen and Iovannisci[27] described a nonisotopic detection method using fluorescently labeled primers. Detection of the LCR product was accomplished using a fluorescent DNA sequencer in conjunction with a GENESCANNER (Applied Biosystems). One of the advantages of this method is that it is relatively easy to quantitate the amount of the LCR products. Furthermore, each of the primers can be labeled with a different fluorescent dye to allow unambiguous assignment of ligation products; incorrect ligation products could be identified by their deviation from the appropriate color combinations.[27] The fluorescent detection system allows multiplexing with the LCR primers specific for a given mutation labeled with different fluorescent tags or with the same fluorescent label and different-sized LCR products.[30] Currently, this method is limited by the requirement for sophisticated equipment. An alternative approach for the nonisotopic detection uses one digoxigenin-labeled primer; the LCR products are detected in a Southern blot format after gel electrophoretic separation.[24]

Recently, more convenient methods for the detection of LCR products in microtiter plates have been developed.[31,32] In this format, one LCR primer of a pair is labeled with biotin at the 5′ end, whereas the other primer is labeled with a nonisotopic reporter at the 3′ end. Reporter groups tested so far include a fluorescein dye in blue (FAM, 5-carboxyfluorescein) and digoxigenin. Direct detection of FAM-labeled LCR products by solution fluorometry showed poor sensitivity, whereas the use of digoxigenin reporter in conjunction with anti-digoxigenin antibodies coupled to alkaline phosphatase (AP) greatly improved the sensitivity. Subsequent detection of the AP could be achieved using colorimetric, fluorescent, or luminogenic substrates. Winn-Deen et al.[31] reported that the luminogenic substrate Lumiphos 530 gave the highest sensitivity in a microtiter plate assay. This sensitivity was only 10-fold less than with detection methods using radioisotopes or a fluorescent DNA sequencer. Another nonisotopic detection method for LCR products has been reported by Zebala and Barany.[33] They utilized primer pairs in which one primer was labeled with a poly(dA) tail at the 5′ end whereas the 3′ end of the other primer was tagged with biotin. The ligated products were captured from the solution via hybridization of their poly(dA) tails with poly(dT)-coated paramagnetic iron beads and subsequent magnetic separation. Only the captured LCR products will carry a 5′-coupled biotin molecule, which can be detected with a streptavidin–AP conjugate and a colorimetric substrate.

For the detection of the products from G-LCR, two different methods have been described. Radioactively labeled nucleotides were used to fill in the gap between the primers, so that the G-LCR products can be detected by autoradiography after gel electrophoresis.[22] Alternatively, the primers can be end-labeled with radioisotopes as described for LCR primers.[21] Nonisotopic detection of G-LCR products was achieved by using pairs of primers labeled with biotin or fluorescein, respectively. Ligated oligonucleotides were captured on antifluorescein-coated microparticles and detected with an antibiotin-AP conjugate. AP activity was subsequently detected with the fluorescent substrate methylumbelliferone phosphate.[23]

CURRENT APPLICATIONS OF LCR

LCR assays have been developed for the detection of genetic diseases as well as for the detection of bacteria and viruses. An overview of the current applications of LCR is shown in Table 3. In many of these applications, LCR is

TABLE 3 Current Applications of LCR and G-LCR

Target	Format	Reference
Genetic diseases		
β-sickle cell hemoglobinemia	LCR, isotopic	Barany[4]
β-sickle cell hemoglobinemia	LCR, fluorescent	Winn-Deen and Iovannisci[27]
Cystic fibrosis	PCR–LDR, fluorescent	Eggerding et al.[15] Winn-Deen et al.[16]
Cystic fibrosis	LCR and G-LCR, isotopic	Fang et al.[34]
Leber's hereditary optic neuropathy	PCR–LCR, nonisotopic	Zebala and Barany[33]
Hyperkalemic periodic paralysis	PCR–LCR, fluorescent	Feero et al.[30] Wang et al.[35]
Bovine leukocyte adhesion deficiency	PCR–LCR, nonisotopic	Batt et al.[25]
Bacteria		
Borrelia burgdorferi	LCR, nonisotopic	Hu et al.[43]
Listeria monocytogenes	PCR–LCR, nonisotopic	Wiedmann et al.[20,32]
Neisseria gonorrhoeae	G-LCR, nonisotopic	Birkenmeyer and Armstrong[23]
Erwinia stewartii	PCR–LCR, isotopic	Wilson et al.[40,41]
Mycobacterium tuberculosis	LCR, fluorescent	Iovannisci and Winn-Deen[42]
Chlamydia trachomatis	G-LCR, isotopic	Dille et al.[22]
Viruses		
Human papillomavirus	LCR, nonisotopic	Bond et al.[46]
Herpes simplex virus	LCR, nonisotopic	Rinehardt et al.[45]
HIV DNA	LCR, nonisotopic	Carrino and Laffler[44]
Other targets		
Ha-*ras* protooncogene	LCR, nonisotopic	Kälin et al.[24]
Ha-*ras* protooncogene	PCR–LCR	Wei et al.[48]
G-6-PD	RT–PCR–LDR, isotopic	Prchal et al.[14]
HOXB7	RT–PCR–LCR, isotopic	Chariot et al.[49]

preceded by an initial PCR step to achieve a greater sensitivity of the respective assays.

Detection of Genetic Diseases

In the initial published reports describing LCR, discrimination between normal β^A- and sickle β^S-globin genotypes in humans was achieved using either an isotopic detection method[4] or fluorescein-labeled LCR primers.[27] Two sets of LCR primers were used, one specific for the normal allele and the other specific for the mutation. These two primer sets were applied in two separate LCR reactions, and the LCR products were analyzed separately. This design allows easy identification of homozygous as well as of heterozygous carriers of the alleles of interest.

Recently, LCR has been exploited for the detection of other mutations responsible for genetic disorders in humans and animals. Examples include cystic fibrosis,[34] Leber's hereditary neuropathy,[33] and hyperkalemic periodic paralysis[30,35] in humans and bovine leukocyte adhesion deficiency (BLAD)[25] in cattle. Screening for the ΔF508, W1282X, and other cystic fibrosis mutations was performed either in two separate LCR reactions targeting the normal and mutant allele or in a "competitive" reaction with six primers, including two common primers, two for the mutant, and two for the normal allele.[34] Detection of alleles leading to hyperkalemic periodic paralysis was achieved by using a multiplex PCR-coupled LCR simultaneously targeting three different potential single base-pair mutations.[30,35] For all of these mu-

tations, LCR primers for the mutant and for the normal allele were included in the LCR, therefore screening for each of these alleles. LCR primers were labeled using a fluorescent dye (FAM) and primers of different lengths so that LCR products for the various mutations and alleles could be differentiated on a fluorescent DNA sequencer by their relative mobility. This approach has recently been extended to the detection of different mutations causing cystic fibrosis.[15,16] Detection of trinucleotide repeats that can give rise to certain diseases, including myotonic dystrophy, has been acheived using repeat expansion detection (RED). In this format, trinucleotide repeat-containing oligonucleotides are ligated when bound in tandem to the target and by cycling greater lengths of these ligation products are generated.[36]

Detection of Bacterial Pathogens

Given the potential of LCR, attempts were made to use this technique for the identification and detection of bacterial pathogens. Detection systems for bacteria based on PCR or other molecular biology techniques usually depend on the availability of well-characterized genus- or species-specific target genes. This strategy is easily applied to extensively documented bacterial pathogens, where the sequence of one or more genes is known. However, for many plant and animal pathogens as well as nonpathogenic bacteria from environmental sources, often there is not sufficient information available to design species-specific PCR primers. The 16S rDNA, encoding part of the ribosomal RNA, consists of both highly conserved and variable regions, the latter usually containing at least single base pair differences that are species-specific. A general method for PCR amplification and sequencing of this gene has been described by Weisburg et al.[37] Our group initially utilized these techniques to sequence the 16S rDNA gene of different isolates of the human pathogen *L. monocytogenes* and the closely related nonpathogenic bacterium *L. innocua*.[38] This method was preferred over direct sequencing of the 16S rRNA using reverse transcriptase, which is not precise enough to identify all nucleotides accurately.[39] After identifying consistent single base-pair differences specific for *L. monocytogenes*, LCR primers were designed to identify this bacterium based on one of these differences. To improve the sensitivity of this LCR, we further employed a set of flanking PCR primers to amplify initially the segment containing the specific single base-pair difference.[20] This PCR-coupled LCR was shown to be highly specific for *L. monocytogenes* and was able to detect, at a minimum, 10 colony-forming units of *L. monocytogenes* using a nonisotopic detection method.[32]

The same approach was used to develop an LCR-based detection method for the plant pathogen *Erwinia stewartii*.[40,41] After sequencing parts of the 16S rDNA gene of *E. stewartii* and the closely related saprophyte *E. herbicola*, *E. stewartii*-specific single base-pair differences were identified. These were again used to design LCR primers for a PCR-coupled LCR, which proved to be specific for *E. stewartii*.

The development of these two PCR-coupled LCR assays for the detection of *L. monocytogenes* and *E. stewartii* suggests that this system is generally applicable for the development of a sensitive detection assay for all bacteria when little or no prior genetic information is available.

Application of LCR for the detection of bacterial pathogens is not limited to targets within the rDNA. Iovannisci and Winn-Deen[42] utilized LCR to detect *Mycobacterium tuberculosis* DNA, based on the insertion sequence IS6110, which is specific for this important pathogen. Using fluorescently labeled primers (ROX, TAMARA, FAM, JOE) and a fluorescent DNA sequencer, it was possible to detect as few as 100 copies of the target molecule even in the presence of unrelated DNA. Furthermore, a nonisotopic LCR for the detection of *Borrelia burgdorferi* has been described.[43]

Assays for the detection of the bacterial pathogens *Neisseria gonorrhoeae* and *Chlamydia trachomatis* using G-LCR have also been described.[22,23] These assays are based on 2-bp differences between the target bacterium and closely related nonpathogenic bacteria. The sensitivity of these assays is approximately one *N. gonorrhoeae* cell using the nonisotopic detection method and three *C. trachomatis* elementary bodies using an isotopic detection method with electrophoretic separation of the products from unligated primers. Detection of *N. gonorrhoeae* was achieved by using G-LCR probes targeting sequences in the gene coding for the cell-surface opacity (Opa) protein or in the gene for the pilin proteins.[23] The targeted sequences show only 2-bp differences between *N. gonorrhoeae* and the closely related *Neisseria meningitidis,* which is sufficient for clear differentiation by G-LCR. For the specific detection of *C. trachomatis,* primers were used that recognized species-specific sequences either in the gene for the major outer membrane protein or on a cryptic plasmid.[22]

Detection of Viruses

Only preliminary reports on the use of LCR for the identification and/or detection of viruses have been published. A nonisotopic ligase-based DNA amplification assay using oligonucleotides targeting part of the *gag* region of HIV-1 has been described. The sensitivity of this assay is between 5 and 10 HIV-1 molecules, which is comparable to the level of sensitivity reached by PCR.[44] LCR technology has also been applied for the nonradioactive detection of herpes simplex virus and HPV and allowed rapid detection of these viruses as compared to traditional detection methods using cell culture techniques.[45,46]

Another ligase-mediated approach for detection of HIV used Qβ-replicase to amplify a target-dependent ligation product of amplifiable hybridization probes. This strategy helps to overcome the problem of target-independent amplification of nonhybridized probes in Qβ replicase assays.[47]

Detection of Other Target Sequences

Kälin et al.[24] described the evaluation of LCR for the detection of single base-pair mutations in the Ha-*ras* proto-oncogene. This group reported a sensitivity of 250 molecules for the targeted mutation but could not differentiate the mutant from the normal allele when a 1:100 ratio of mutant to normal DNA was used. These problems might be caused by the use of LCR primers with a blunt end rather than a single base-pair overhang, which is known to cause higher target-independent ligation (see above, under Design of LCR Primers). Wei et al.,[48] on the other hand, were successful in developing a combination of PCR and LCR for the detection of point mutations in the Ha-*ras* proto-oncogene. Using two cycles of *Msp*I restriction, PCR amplification, and a subsequent LCR amplification, they were able to detect mutant in a background of 10^8 wild-type alleles.

Prchal et al.[14] used a combination of RT–PCR and LDR for transcriptional analysis to determine the active X-chromosome based on a polymorphic locus on this chromosome. In the first step, DNA isolated from a person is tested for heterozygosity in the target allele using a PCR-coupled LDR. Only persons found to be heterozygotic are then subjected to transcriptional analysis. For this purpose mRNA is isolated from the cells of interest, for example, lymphocytes, myeloid cells, and fibroblasts, and used for RT–PCR amplification and subsequent LDR. The LDR then detects the allele transcribed in the isolated cells, therefore indicating the clonality of these cells. This assay can be applied for the specific and sensitive determination of clonality in cells,

cell linages, and tissues, which is important for studies of neoplastic disorders and embryologic development.

Another application of a cancer-related mutation using a RT–PCR-coupled LCR has been described by Chariot et al.[49] This group used LCR to detect the expression of stop codon polymorphism in the homeo domain sequence HOXB7 of a breast cancer-derived cell line. This work demonstrates the potential of RT–PCR-coupled LCR in RNA diagnostic procedures by the example of the sensitive detection of single-base polymorphisms in rare mRNA transcripts.

OUTLOOK

With the continuing emergence of sequence data for the human genome as well as the genomes of other species (e.g., bovine, equine), the potential of LCR to detect genetic diseases that result from single base-pair mutations is immense. One of the inherent advantages of LCR is its potential for automation. The LCR product consists of two covalently joined primers that can be easily detected using different enzyme-linked or direct fluorescent labels. Formatting of multiplex LCR assays will further improve screening samples for an array of different single base-pair changes in a single tube. Automated, multiplex LCR or PCR-coupled LDR/LCR assays have a variety of potential applications, such as[5] (1) screening of large populations for monogenic disease polymorphisms; (2) determining HLA haplotypes in tissue typing, for example, for transplantation; and (3) screening for multiple bacterial species after a generic PCR amplification of 16S rDNA sequences.

In clinical diagnosis of pathogenic bacteria and viruses, the specificity of LCR could be useful in many applications. The detection of single base-pair differences in bacterial pathogens may be valuable with respect to antibiotic resistance arising from point mutations, for example, in some cases of macrolide resistance[50] or from transformational exchange as occurs in sensitive and resistant strains, for example, in *N. meningitidis.*[51] In viral pathogens, the identification of subpopulations with genetic differences may be important with regard to host range, virulence characteristics, and drug resistance.

Furthermore, the application of LCR and PCR-coupled LCR assays for the detection of specific bacteria based on at least a single base-pair difference in the 16S rDNA gene has great potential. As outlined above, such a system circumvents the need to identify species-specific genes, as warranted for PCR or other nucleic acid-based assays. With emerging interest in yet poorly characterized bacteria, this method should have a great potential as a detection system.

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