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The Ligase Chain Reaction in a PCR World

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Since its discovery in 1985, the polymerase chain reaction (PCR) has had a profound impact on detecting genetic and infectious diseases, identifying new genes, and unraveling the mysteries of protein–ligand recognition.^(1–5) Its universal utility is due to the exquisite specificity of amplification and ease of cycling made possible by the cloning and careful characterization of a thermostable polymerase from *Thermus aquaticus*.^(6,7) Likewise, cloning of a thermostable ligase enabled a new amplification method, termed ligase chain reaction (LCR), to both amplify DNA and discriminate a single base mutation.^(8–10) Although these DNA amplification techniques are new, they bring to fruition the "enzymes as reagents" philosophy expounded by A. Kornberg and I.R. Lehman a quarter of a century ago.

Allele-specific LCR employs four oligonucleotides, two adjacent oligonucleotides which uniquely hybridize to one strand of target DNA and a complementary set of adjacent oligonucleotides, which hybridize to the opposite strand (see Fig. 1, top left). Thermostable DNA ligase will covalently link each set, provided that there is complete complementarity at the junction.⁽⁸⁾ Because the oligonucleotide products from one round may serve as substrates during the next round, the signal is amplified exponentially, analogous to PCR amplification. A single-base mismatch at the oligonucleotide junction will not be amplified and is therefore distinguished (Fig. 1, top right). A second set of mutant-specific oligonucleotides is used in a separate reaction to detect the mutant allele.

This review will give a brief introduction to (1) determinants of specificity in amplification reactions; (2) differences between thermostable and thermophilic enzymes; (3) detec-

tion of single-base substitutions in DNA diagnostics; (4) characterization of DNA ligases; (5) cloning of DNA ligases; (6) use of ligases in DNA detection; (7) other methods that use ligase, such as ligase-mediated PCR and the branch capture reaction; and (8) potential new uses of ligase in PLCR and nested LCR amplification reactions. It will close with speculations on future prospects.

SPECIFICITY, THERMOSTABILITY, AND THERMOPHILIC ORGANISMS

PCR amplification exploits two primers to obtain three types of information: (1) presence of target sequence, (2) distance between primers, and (3) sequence in between the primers. LCR exploits four primers to obtain only two types of information: (1) presence of adjacent target sequences, and (2) presence of perfect complementarity to the primers at the junction of these sequences. Both LCR and PCR amplification derive their specificity from the initial hybridization of primer to target DNA. This specificity is enhanced by: (1) use of oligonucleotides of sufficient length to uniquely identify individual humans or the target genome, and (2) use of temperatures near the oligonucleotide T_m . With PCR, background target-independent amplification results in primer dimers, which are of lower molecular weight and thus easily distinguished. However, with LCR, background target-independent amplification yields the *same* size product. Hence, to reduce LCR to practice, *it was necessary to eliminate target independent ligations completely*. This was accomplished with use of a thermostable ligase.^(8–10)

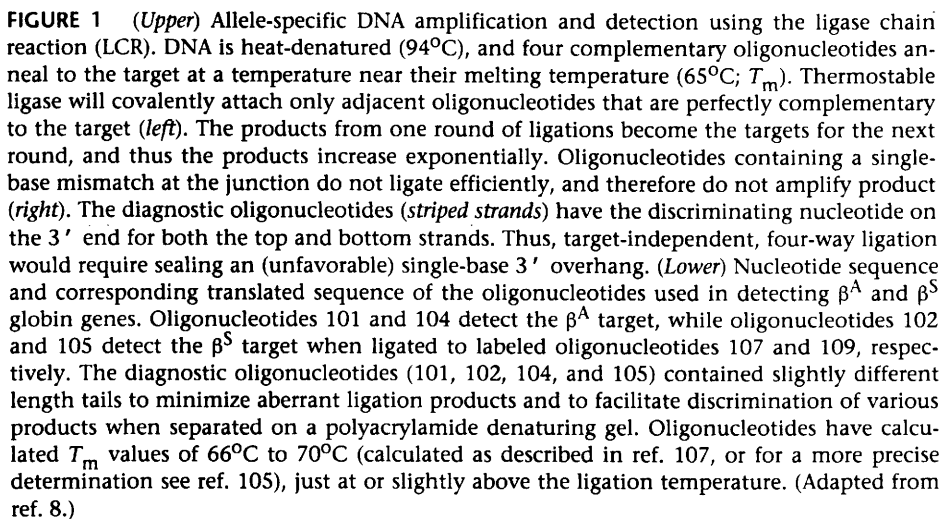
For an effective amplification reaction to take place, a *thermostable* enzyme must not become denatured irreversibly when subjected to the elevated temperatures (about 90–100°C) for the

amount of time necessary to effect complete denaturation of double-stranded DNA (about 30–60 sec). Both *Taq* polymerase and *Taq* ligase retain activity after 20 or 30, or more, repeated 1-min exposures to 94°C,^(6,8) and hence are termed *thermostable*. The *Taq*I restriction endonuclease, isolated from the same thermophilic *T. aquaticus* species, does not survive such treatment (being completely inactivated after 20 min at 85°C⁽¹¹⁾), and hence is termed a *thermophilic* enzyme.

T. aquaticus YT1⁽¹²⁾ and *T. thermophilus* HB8⁽¹³⁾ were isolated originally on two different continents. Currently, they are classified by the American Type Culture Collection (ATCC) as a single species.⁽¹⁴⁾ The amino acid sequences of *T. aquaticus* YT1 and *T. thermophilus* HB8 restriction endonucleases,^(15–17) methylases,^(15–17) and DNA polymerases⁽¹⁸⁾ show 77, 79, and 88% identity, respectively, and DNA homology studies of numerous thermophilic isolates suggest that these organisms may indeed be separate species.⁽¹⁹⁾ Until the taxonomy is resolved, both designations are correct and will be used interchangeably.

DNA DIAGNOSTICS

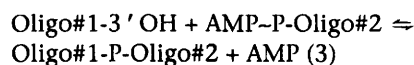
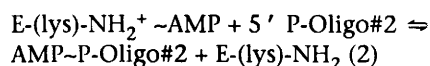
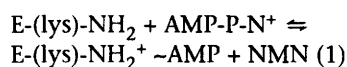
DNA diagnostics employs the tools of molecular biology to detect the presence of bacterial and viral infectious agents, genetic traits, and diseases.⁽²⁰⁾ Furthermore, DNA diagnostics can uniquely identify humans, animals, and plants.⁽²⁰⁾ This often demands exquisite specificity to distinguish closely related (drug-resistant vs. -sensitive) pathogens or single-allele diseases, which may consist of subtle deletions, insertions, or single-nucleotide substitutions in over 3 billion base pairs of target DNA. A reliable DNA diagnostics method requires



(3SR),⁽²¹⁾ or Q- β replicase-mediated RNA amplification.⁽²²⁾ Subsequently, small deletions, insertions, or single-base mismatches may be detected through use of allele-specific oligonucleotide hybridization,^(23,24) reverse-oligonucleotide blot-dot hybridization,⁽²⁵⁾ denaturing gradient gel elec-

In the late 1960s, models of DNA recombination, replication, and repair proposed the existence of an enzyme that could form a covalent phosphate link between two strands of DNA. No less than five separate groups, using five separate assays, independently and virtually simultaneously, announced the discovery of such an enzyme, termed DNA ligase.⁽³⁷⁻⁴⁴⁾ DNA ligase uses either an ATP (T4 enzyme) or NAD (*Escherichia coli* enzyme) cofactor to join covalently the adjacent 3' hydroxyl and 5' phosphoryl termini of nucleotides that are perfectly hydrogen bonded to a complementary strand. Substrate assays for this reaction include: (1) circularization of linear "sticky end" phage λ DNA to a form that does not denature at alkaline pH,^(37,38) (2) resistance of 5' ³²P-labeled oligo(dT₁₅₀) to alkaline phosphatase treatment after ligation in the presence of complementary strand poly(dA₄₀₀₀),^(39,40) (3) covalent capture and alkaline-resistant precipitation of labeled oligo(dC) after ligation to cellulose-oligo(dC) in the presence of complementary poly(dI),⁽⁴¹⁾ and (4) resistance of labeled poly(dAT) to exonuclease III degradation after forming a self-complementary closed circular loop.⁽⁴⁵⁾ The ligation reaction occurs in three discrete and reversible steps: (1) formation of a high-energy enzyme intermediate by transfer of the adenosyl group from NAD (or ATP) to the ϵ -amino group of a lysine residue; (2) transfer of the adenosyl group to the 5' phosphate of one DNA strand thus forming an activated pyrophosphate linkage; and finally (3) attack of this activated 5' end by a 3'-hydroxyl group on the adjacent DNA strand,

thus forming a phosphodiester link between the two DNA strands, and eliminating AMP.⁽⁴⁶⁻⁵⁴⁾ These steps are shown below for the *E. coli* DNA ligase.



The ligase-adenylate intermediate was so stable that it could be readily identified and separated by acid precipitation of radioactively labeled enzyme, gel filtration, or its slower mobility after electrophoresis through an SDS-polyacrylamide gel.^(47,49,50,54) Proteolytic degradation of the adenylated adduct revealed adenosine 5' monophosphate linked to the ϵ -amino group of lysine.⁽⁴⁶⁾ This phosphamide linkage could be reversed by exchanging with NMN or PP_i in the absence of DNA substrate, or alternatively transferred to the 5' phosphate of a DNA substrate. Formation of a ligase-adenylate intermediate has been used to identify and clone two yeast ligase genes.⁽⁵⁵⁻⁵⁷⁾

The T4 and *E. coli* enzymes exhibit slightly different activities; only the *E. coli* enzyme is activated by ammonium ions (about 20-fold), whereas the T4 enzyme has higher activity for blunt ends or RNA-DNA hybrids.⁽⁴²⁾ Blunt-end activity may be readily detected for the *E. coli* enzyme when using molecular-crowding agents such as PEG,⁽⁵⁸⁾ or appropriately sensitive assays.⁽⁵⁹⁾ *T. thermophilus* HB8 ligase shows only a very slight stimulation by ammonium ions,⁽⁶⁰⁾ and like its *E. coli* homolog, displays blunt-end activity in the presence of PEG, even at 65°C.⁽⁶¹⁾ As observed with the *E. coli* enzyme, the thermostable ligase-adenylate intermediate migrates with an apparent molecular weight of about 81,000, as compared with about 78,000 for the nonadenylated form on an SDS-polyacrylamide gel.⁽⁶²⁾

CLONING OF DNA LIGASES

Most molecular biologists use huge excesses of DNA ligase for cloning countless exotic genes, unaware of the im-

pact that cloning the actual T4 and *E. coli* ligase genes themselves had on the early days of biotechnology. This cloning has been greatly facilitated by the characterization of two temperature-sensitive mutants of the *E. coli* gene.⁽⁶³⁻⁶⁵⁾ The *ligts4* strain contained normal levels of enzyme at 25°C and only 1% at 42°C, whereas the *ligts7* strain had only 1% enzyme at 25°C, and lost viability at 42°C. Neither strain supported growth of an integration-deficient (Red⁻) λ phage, and the combination of this host-vector system provided an elegant positive selection of recombinant phages containing the T4 or *E. coli* ligase gene.⁽⁶⁶⁻⁶⁸⁾ Overproduction of the enzyme reduced purification to a simple three-step procedure.^(69,70) DNA sequence analysis of the *E. coli* gene revealed a single chain of 671 amino acid residues,⁽⁷¹⁾ with no significant identities to the T7, T4, or yeast ligases.^(56,57,72,73)

Thermostable ligase was cloned by screening for growth of a *ligts7* derivative (AK76) at 42°C, when complemented with plasmid libraries of *T. aquaticus* HB8 DNA.^(8,10) To assure that the highly methylated *T. aquaticus* HB8 DNA (including TCGA and AATT sites⁽¹⁵⁾) did not undergo *mrr*-associated restriction or mutation,⁽⁷⁴⁾ it was necessary to prepare libraries in an *E. coli* host strain (such as AK76) lacking not only *mrrA*, but, more importantly, a newly discovered methyl-dependent endonuclease termed *mrrB*.^(8,15,62) True complementation by a plasmid containing the thermostable ligase gene (pDZ1) could be distinguished from revertants by pinpoint colony size, as well as presence of a thermostable NAD-dependent nick-closing (DNA ligase) activity in crude extracts when assayed at 65°C. Furthermore, DNA sequence analysis of the first 60 codons of the putative gene revealed >50% amino acid identity to *E. coli* ligase.^(8,62) Enzyme overproduction was achieved by replacing the endogenous transcription and translation signals with a *phoA* promoter-ribosome binding site.^(62,75) A heat treatment step during purification (65°C for 20 min⁽⁷⁶⁾) allowed for rapid production of exo- and endonuclease-free thermostable ligase with a specific activity of 1.6 million (nick-closing)

units/mg.⁽⁶²⁾ DNA sequence analysis of the *T. thermophilus* ligase gene revealed a single chain of 676 amino acid residues with 47% identity and 66% similarity to the *E. coli* ligase gene.⁽⁶²⁾ A similar thermophilic gene also has been cloned by screening for a heat-stable, ligase-adenylate intermediate,⁽⁷⁷⁾ essentially as described previously for the yeast ligase genes.^(56,57)

LIGASE-MEDIATED DNA DETECTION

From the variety of exceedingly sensitive assays first described for ligase, it was readily apparent that this enzyme could serve as a reporter for the presence of two adjacent strands of DNA hybridized to a complementary target DNA strand.^(37-41,45) As part of a herculean effort to achieve total synthesis of tRNA genes, the adjacent position of two oligonucleotides could be proved by the ability of T4 ligase to link these oligonucleotides only when hybridized to the r strand of ϕ 80psu⁺ DNA.⁽⁷⁸⁾ A dozen years passed before synthetic oligonucleotides were readily available, allowing for practical use of this concept. This assay has been used to detect the presence of minute quantities of ligase in crude preparations,⁽⁷⁹⁾ or to detect the presence of sub-picomole quantities of λ phage DNA, and has clear diagnostic implications.^(80,81) Landegren et al. pioneered the use of such an oligonucleotide ligation assay (OLA) to detect single nucleotide substitutions in both cloned and clinical samples.^(59,82-89) Use of a biotin "hook" on the first probe and a suitable non-isotopic reporter group on the second probe allowed for product capture (with streptavidin) and detection, thus circumventing the need for electrophoresis or precise hybridization conditions.⁽⁸²⁾ The method has been combined with a primary PCR amplification to screen for sickle cell anemia, cystic fibrosis, α_1 -antitrypsin deficiency, and T-cell receptor polymorphisms in an automated ELISA-based format.^(83,85)

Thermostable ligase discriminated single-base mismatches under both LDR (ligase detection reaction; using two adjacent probes) and LCR (ligase chain reaction; using two pairs of adjacent probes) conditions, with a

signal-to-noise ratio ranging from 75 to greater than 500.⁽⁸⁾ With no background over a range of salt conditions, this represented a substantial improvement in specificity, sensitivity, and flexibility when compared with use of mesophilic enzymes.^(59,87,88,90) Furthermore, for amplification reactions, mesophilic ligases were unable to eliminate target-independent (blunt end) background ligation, thus limiting practical use.^(59,88,90) Four-way (target-independent) ligation was minimized in LCR with thermostable ligase by the following procedures: (1) addition of 4 μ g of carrier salmon sperm DNA, (2) use of single-base 3' overhangs on discriminating oligonucleotides, (3) 5' phosphorylation of the adjacent (ligating) oligonucleotides only, (4) presence of noncomplementary tails on the outside of oligonucleotides, and (5) use of cycling conditions at or near the oligonucleotide T_m .⁽⁸⁾ Omission of either carrier salmon sperm DNA or use of "blunt-end" oligonucleotides gave detectable background signal.^(8,91) The method could detect 200 target molecules, and could discriminate between normal β^A - and sickle β^S -globin genotypes from 10- μ l blood samples, as shown in Figure 2.⁽⁸⁾ The efficiency of ligation (and hence detection) was somewhat less for β^S -

than for β^A -specific oligonucleotides, when either plasmid or human chromosomal target DNA was used, with either radioactive⁽⁸⁾ or fluorescent detection.⁽⁹²⁾ This difference may be a function of the exact nucleotide sequence at the ligation junction, and, although subtle, is amplified multiple times. Thus, about two- to threefold more product was detected for β^A than for β^S target DNA. Such differences should be considered when designing assays wherein two allele-specific oligonucleotides compete for ligation to an adjacent invariant oligonucleotide. Optimized LCR conditions for detecting sickle cell anemia, *Mycobacterium tuberculosis*, and human papillomaviruses are presented in Table 1.^(8,92-94)

LIGASE-MEDIATED PCR AND THE BRANCH-CAPTURE REACTION

Ligase-mediated PCR combines the specificity of hybridizing to a single primer site with (nonspecific) blunt-end linker ligation to amplify and identify sequences where only one side is known.^(95,96) Chromosomal DNA is nicked or cleaved in a defined way, the DNA denatured, and a specific primer extended with Sequenase to generate a new blunt end. A unidirectional (staggered) unphosphorylated linker is at-

tached only to these newly synthesized blunt ends with T4 ligase. Both a second primer, longer and downstream from the first, and the unidirectional linker primer are then used to amplify the appropriate fragments specifically. Products are identified by either Southern blotting or by extending with a third end-labeled primer, and fragments are analyzed on a denaturing polyacrylamide sequencing gel. This method has been successfully applied to genomic sequencing,⁽⁹⁷⁾ genomic DMS footprinting,⁽⁹⁸⁾ genomic methylation studies,^(97,99,100) and cloning of promoter elements.⁽¹⁰¹⁾ For cloning purposes, use of a non-complementary unidirectional linker (i.e., lacking a palindromic restriction site on the 3' end) or a vectorette-linker with an internal "bubble",⁽¹⁰²⁾ would probably reduce formation of aberrant PCR products.

In a branch-capture reaction (BCR), a displacer strand is used to bring a complementary recipient duplex strand 3' end adjacent to the 5' end of a complementary linker strand, creating a substrate for covalent linkage by ligase.⁽¹⁰³⁻¹⁰⁵⁾ Use of 5Met-dCTP in an asymmetrically synthesized displacer and thermostable ligase greatly enhanced the specificity of DNA capture.⁽¹⁰⁴⁾ The branch-capture reaction may also be used as an alternative to biotin/streptavidin as a means of capturing specific LCR-, OLA-, or PCR-generated product for detection.^(8,20,80-85,94) Combining the branch-capture reaction (steps 1-3, Fig. 3) with ligation-mediated PCR (steps 4-8, Fig. 3) provides the potential for specifically capturing and amplifying any chromosomal DNA fragment.

PLCR, NESTED LCR, AND OTHER VARIATIONS

LCR amplification aims to discriminate accurately between different alleles while achieving the highest signal-to-noise ratio. For example, LCR did not amplify a T-T, G-T, C-T, or C-A 3' terminal mismatch,⁽⁸⁾ as has been reported for some allele-specific PCR amplifications.⁽³²⁾ One approach to increase the specificity of allele-specific PCR is to use low (1 μ M) concentrations of dNTPs, although this would limit the extent of amplification.⁽¹⁸⁾ Allele-specific addition of one or two

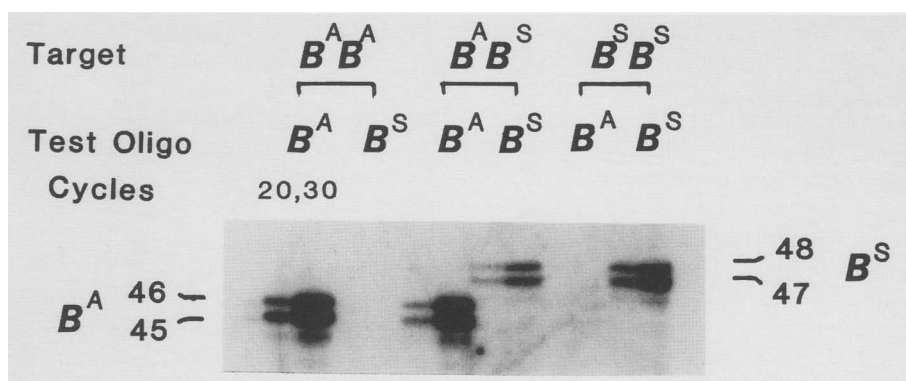


FIGURE 2 Autoradiogram showing detection of β -globin alleles in human genomic DNA. DNA was isolated from blood samples from normal ($\beta^A \beta^A$), carrier ($\beta^A \beta^S$), and sickle cell ($\beta^S \beta^S$) individuals as described in ref. 8. Genomic DNA (corresponding to 10 μ l of blood, or about 6×10^4 nucleated cells) was tested in two separate tubes containing labeled oligonucleotides (107 and 109; 200,000 cpm = 40 fmoles each), and either unlabeled β^A test oligonucleotides (101 and 104) or unlabeled β^S test oligonucleotides (102 and 105; 40 fmoles each). Both reaction mixtures were incubated under the same buffer (without salmon sperm DNA), enzyme, and cycle conditions described in method 1 (Table 1). Samples were electrophoresed in a 10% polyacrylamide denaturing gel, and subjected to gel autoradiography overnight, as described in ref. 8. Ligation products of 45 and 46 or 47 and 48 nucleotides indicate the presence of the β^A or β^S globin gene, respectively. (From ref. 8, with permission.)

TABLE 1 Ligase Chain Reaction Methods

	Method 1 ^a	Method 2 ^a	Method 3 ^a
Target DNA	β^A, β^S	β^A, β^S	human papillomavirus
Standard detection	1–10 attomoles (6×10^5 to 6×10^6 molecules)	10^5 – 10^6 molecules	10^6 molecules
Signal-to-noise no target under standard conditions	1700 to >2000 ^b	20–30	26
single-base mismatch under standard conditions	75 to >500 ^b	18–30	not done
Lowest detection	200 molecules	1000 molecules	500 molecules
Position of discriminating nucleotide	3' base of both strands (single- base 3' overhang)	3' base of both strands (single- base 3' overhang)	not done
T_m discrimination oligonucleotides	64°C–68°C (23- to 28-mers)	62°C–64°C (20- and 21-mers)	not reported
T_m adjacent oligonucleotides	70°C (22-mers)	60°C–62°C (19- and 20-mers)	not reported
Amount of each oligonucleotide	40 femtomoles (0.28 ng)	0.5–1 picomole (3.5–7 ng)	1–1.5 picomole (7–10 ng)
Volume	10 μ l	100 μ l	50 μ l
Buffer conditions	20 mM Tris-HCl, pH 7.6 ^c 100 mM or 150 mM KCl 10 mM MgCl ₂ 10 mM DTT 10 mM NAD ⁺ 1 mM EDTA	20 mM Tris-HCl, pH 7.6 100 mM KCl 10 mM MgCl ₂ 10 mM DTT 1 mM NAD ⁺ 0.1% Triton X-100	50 mM EPPS, pH 7.8 100 mM K (as OH [−] and Cl [−]) 10 mM MgCl ₂ 1 mM DTT 0.1 mM NAD ⁺ 10 mM NH ₄ Cl 10 μ g/ml BSA
Carrier DNA to suppress background	4 μ g of salmon sperm DNA	4 μ g of herring sperm DNA	not reported
Additional features for suppression of target independent background	5' phosphate on adjacent oligonucleotides only; noncomplementary tails on outside of oligonucleotides; single-base 3' overhang on discriminating oligonucleotides	fluorescent dye label on nonligating ends of all four oligonucleotides; single-base 3' overhang on discriminating oligonucleotides	fluorescent and biotin moieties on nonligating ends
Thermostable enzyme	15 nick-closing units ^d	15 nick-closing units	not reported
Cycle conditions	94°C, 1 min 65°C, 4 min 20 or 30 cycles or 94°C, 0.5 min 65°C, 2 min 30 or 40 cycles	94°C, 1 min 62°C, 2 min (5-sec autoextend/cycle) 30 or 40 cycles	85°C, 0.5 min 50°C, 0.35 min 30 or 35 cycles

^a Method 1⁽⁸⁾ used radioactive detection with direct quantitation of products. Method 2⁽⁹²⁾ and method 3⁽⁹⁴⁾ used fluorescent detection essentially as described by Landegren et al.⁽⁸²⁾ Method 2 has also been applied to detection of *Mycobacterium tuberculosis*.⁽⁹³⁾

^b Signal-to-noise ratios may be better than indicated for method 1, since no incorrect product was observed at the limit of detection.⁽⁸⁾

^c pH value determined at 25°C.

^d One nick-closing unit of ligase is defined as the amount of ligase that circularizes 0.5 μ g of DNase I-nicked pUC4KIXX DNA (about 10–20 nicks per plasmid) in 20 μ l of 20 mM Tris-HCl, pH 7.6 (at 25°C), containing 50 mM KCl, 10 mM MgCl₂, 1 mM EDTA, 10 mM NAD⁺, 10 mM dithiothreitol, overlaying with a drop of mineral oil, after a 15-min incubation at 65°C.⁽⁸⁾

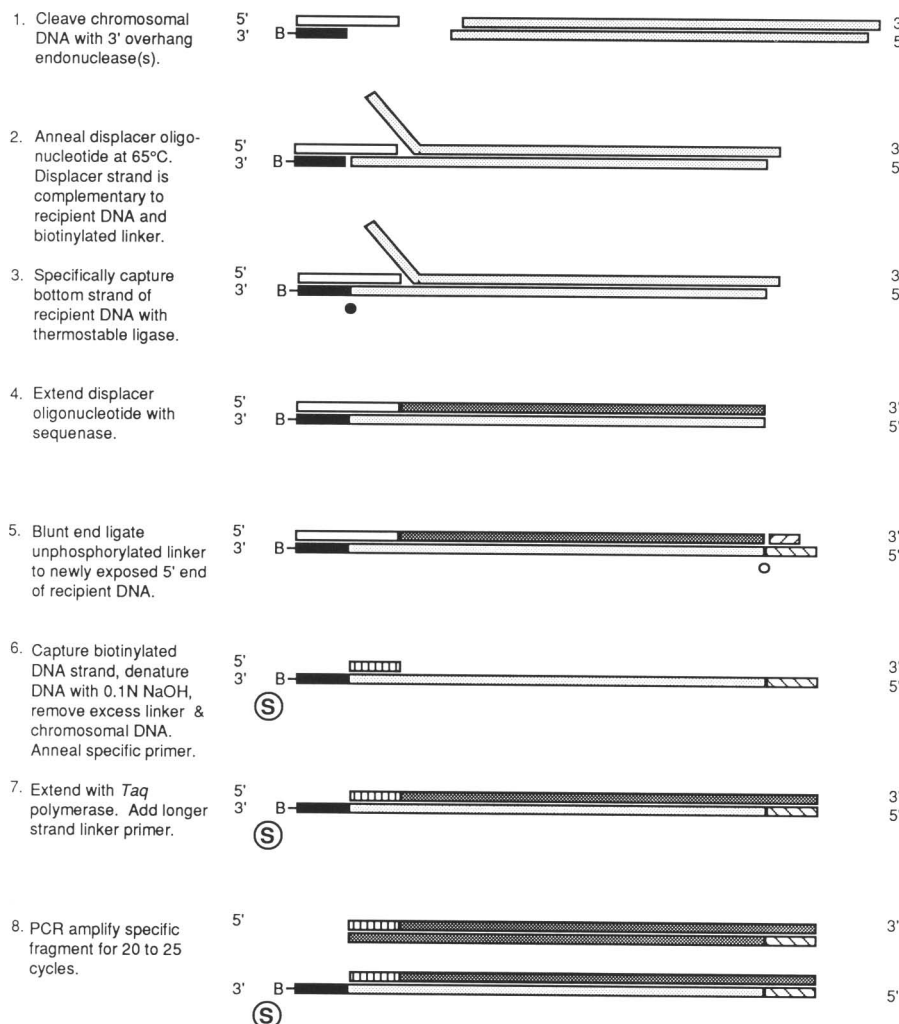


FIGURE 3 A diagram depicting use of a combination of the branch-capture reaction and ligase-mediated PCR to clone any chromosomal DNA fragment (BCLPCR). After cleaving chromosomal DNA (*lightly shaded strands*) with one or more restriction endonucleases (preferably generating a 3' overhang), a displacer strand (*white strand*), partially complementary to the end of the recipient strand, and partially complementary to a linker strand, and a biotinylated linker (*black strand*) complementary to the displacer is added. The displacer strand may be either chemically synthesized, or generated via asymmetric PCR, and contains modified cytosines (5MedC or 5BrdC) to increase the oligonucleotide T_m above that of the recipient displaced strand. By incubating the reaction at 65°C in the presence of thermostable ligase, the displacer strand should specifically direct capture of only complementary recipient strands by covalent linkage to the linker. Upon extending the displacer strand with Sequenase (*darkly shaded strand*), the newly generated blunt end provides a suitable substrate for attachment of an unphosphorylated unidirectional linker (*striped strands*). Excess linker, chromosomal DNA, and enzymes are conveniently removed by capturing the biotinylated DNA fragment with streptavidin-coated magnetic particles (Dynal), and washing in 0.1 N NaOH. After neutralization, a second specific primer (*horizontal striped strand*) slightly 3' to the displacer oligonucleotide is annealed and extended with *Taq* polymerase. Addition of the longer unidirectional linker oligonucleotide followed by PCR will specifically amplify the desired fragment. (Adapted from refs. 95, 96, and 103–105.)

nucleotides using the *Taq* polymerase Stoffel fragment (lacking not only the 3'–5' proofreading exonuclease, but also the 5'–3' nick-translation exonuclease⁽¹⁰⁶⁾) could fill a gap between

two oligonucleotides, which would be sealed with *Taq* ligase, in a process termed PLCR (see Fig. 4). This would simultaneously increase allele-specific discrimination while avoiding target-

independent ligation because the oligonucleotide pairs are no longer adjacent to each other.

An alternative approach for further improving signal-to-noise is use of a primary LCR amplification to generate the primers required for a secondary allele-specific LCR amplification (nested LCR, see Fig. 5). Two separate tubes are used in the primary amplification, each containing shorter LCR primers (about 15 nucleotides in length, with T_m values around 55°C, calculated as described in ref. 107, or for a more precise determination see ref. 105) corresponding to about 30 nucleotides flanking the left and about 30 nucleotides flanking the right of the allele-specific base pair, respectively. LCR amplification (cycling between 55°C and 94°C) will form significant full-length primer products (30 nucleotides) only in the presence of target DNA. The resultant primers are combined, and fresh ligase and an organic solvent such as ethylene glycol, glycerol, formamide, or DMSO is added (depressing oligonucleotide primer T_m values to about 85°C.^(104,108,109)). A second LCR amplification (cycling between 85°C and 94°C) will specifically form a full-length product (60-mer) only if the correct allele target DNA is present. Nested LCR should further minimize target-independent product formation.

Thermostable ligase may also be used to covalently link two members of a hexamer oligonucleotide library to form specific dodecamers for directed sequencing of cosmid and other large DNAs.^(110–113) Initially, randomly selected nonamers would be used to start sequencing randomly throughout a cosmid clone.^(110,113) By judicious choice of two or four hexamers from a 2994-member library, one could generate a dodecamer primer through either target-dependent LDR amplification, or target-dependent or -independent LCR amplification using 2-base 3' overhangs among the four oligonucleotides.^(111,114,115) Improper priming may be minimized by: (1) using *Taq* polymerase to extend dodecamer primers at 37 or 45°C, temperatures well above the hexamer T_m ; and (2) using one hexamer primer with a 2',3'dideoxynucleotide to block extension off the incorrect LCR dodeca-

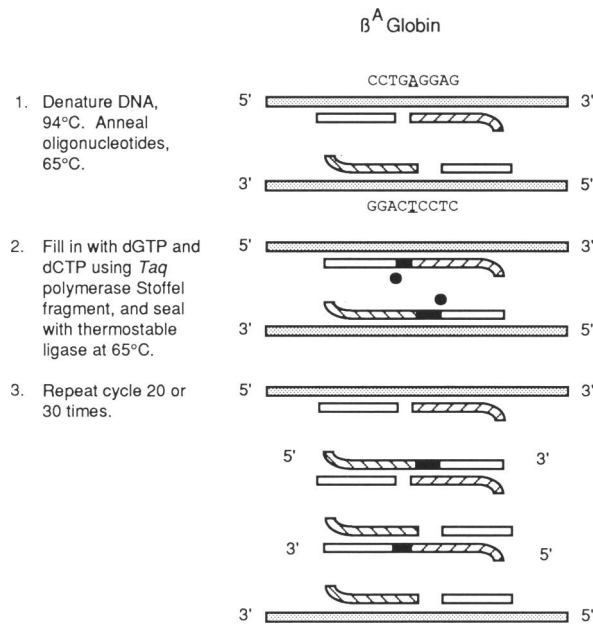


FIGURE 4 Allele-specific DNA amplification and detection using the *Taq* polymerase Stoffel fragment and *Taq* ligase (PLCR). DNA is heat-denatured (94°C) and four complementary (but not adjacent) oligonucleotides anneal to the target at a temperature near their melting temperature (65°C). The diagnostic oligonucleotides (striped strands) contain the discriminating nucleotide on the 3' end. The *Taq* polymerase Stoffel fragment, which lacks the 5'–3' exonuclease, is added in the presence of 1 μ M dGTP and dCTP to fill the gap between oligonucleotides (black), provided the last nucleotide is complementary to the target DNA. Thermostable ligase would subsequently covalently link only the extended diagnostic oligonucleotide to the newly adjacent (white strand) oligonucleotide. The specificity of this type of amplification depends on the fidelity of polymerase extension at a mismatch.

mer primer.⁽¹¹¹⁾ Sequences would be extended in a directional manner, using ligase to synthesize unique dodecamer primers, until sequences on both strands overlap the entire cosmid clone.

FUTURE PROSPECTS

Perhaps the greatest potential for LCR amplification and detection is its compatibility with a primary amplification of genomic DNA or RNA by either PCR⁽⁶⁾ or 3SR.⁽²¹⁾ One can envisage multiplexing the primary amplification of dozens of loci simultaneously,⁽¹¹⁶⁾ and aliquoting products into separate microtiter wells. A subsequent round of LCR amplification and detection could then distinguish a particular target loci, even if it were initially amplified in only the attomolar range. LCR amplification is compatible with radioactive, chemiluminescent, fluorescent, or enzymatic reporter groups.^(8,92–94) LCR amplification creates a covalent link between oligonucleotides; use of a capture group on one

primer and a reporter group on the adjacent primer gives the advantage of allowing for automation with high throughput, as has been elegantly demonstrated with the analogous OLA detection procedure.^(83,85) Such an automated multiplex PCR/LCR detection assay could: (1) rapidly screen large populations for monogenic disease polymorphisms,^(83,85) (2) determine HLA haplotypes for tissue typing and transplantation^(117–120) (3) help distinguish single base deletions in poly(A) or poly(G) tracts that are not amenable to allele-specific PCR,⁽¹²¹⁾ (4) distinguish several polymorphisms simultaneously from a single sperm to map the relative positions of these polymorphisms,^(33,122) and (5) help eliminate current ambiguities in DNA identification of individuals for forensic or paternity cases.⁽¹²³⁾

The most crucial determinant in a clinically useful disease detection assay is the signal-to-noise ratio for distinguishing two alleles differing by a

single base pair. For example, among the infectious diseases, it is important to differentiate pathogenic agents in the presence of nonpathogenic normal flora, or to detect emerging viral subpopulations where the mutations are known, such as the multiple mutations in HIV conferring resistance to AZT.⁽¹²⁴⁾ LCR amplification currently has a signal-to-noise ratio of from 75 to greater than 500,⁽⁸⁾ and can thus theoretically detect a pathogenic or resistant subpopulation of from 7% to 1% with a fivefold higher signal than for the nonpathogenic or sensitive population signal. Whether LCR will tolerate internal mismatches between the primers and (HIV) viral variants,^(32,125) while still maintaining high signal-to-noise ratios, remains to be determined.

One potential roadblock in developing prenatal diagnosis for monogenic diseases is the large variety of mutations present in the human population. As of 1990, over 90 point mutations producing β -thalassemia have been catalogued, and these include frameshift mutations, nonsense codons, small deletions, transcription defects, RNA splicing defects, capping site mutations, RNA cleavage defects, initiator codon changes, and unstable transcripts.⁽¹²⁶⁾ Methods to identify the precise sequence of newly arising mutations rapidly have been recently developed.^(29,34,127–129) LCR amplification has the potential to detect a vast number of mutations through a different type of multiplexing format. The entire coding region of the target gene would be amplified as a set of PCR fragments that bracket all known mutations. LCR amplification of several known mutations could be tested in a single tube. Again, with a current signal-to-noise ratio of from 75 to greater than 500, one could theoretically detect the presence of one disease-carrying allele while simultaneously testing from 15 to 100 potential mutations, with a fivefold higher signal than for the combined wild-type signal of all those alleles tested. Improving the allele-specific LCR signal-to-noise ratio and use of several non-overlapping reporter groups (i.e., fluorescent detection) could ultimately allow for rapid testing of several hundred polymorphic disease mutations in a given gene.

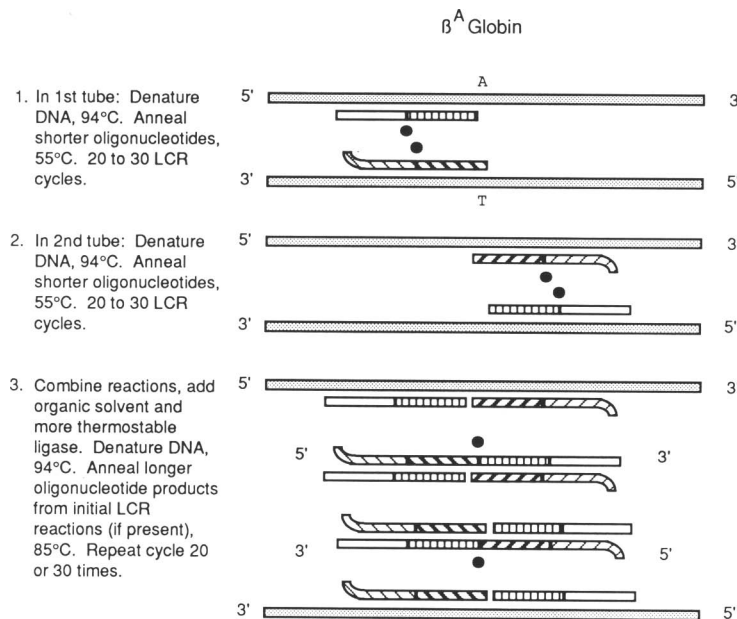


FIGURE 5 Allele-specific DNA amplification and detection using two levels of the LCR nested LCR). The first tube contains two sets of adjacent oligonucleotide pairs, about 15 nucleotides in length, which anneal to the left of the allele-specific nucleotide. LCR amplification is performed by cycling between 94°C and the oligonucleotide melting temperature (about 55°C). The oligonucleotides are designed with single-base 3' overhangs and noncomplementary ends on one side. The second tube contains an analogous set of four oligonucleotides to the right of the allele-specific nucleotide. The LCR products are combined, and ethylene glycol, glycerol, formamide, or DMSO is added to depress oligonucleotide primer product T_m values to about 85°C. With fresh ligase added, a new round of amplification is performed at the 30-mer melting temperature of about 85°C. The diagnostic primer product oligonucleotides (30-mer; lightly and strongly striped strands) have the discriminating nucleotide on the 3' end for both the top and bottom strands. The full-length (60-mer) product forms only if the correct allele target DNA is present.

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scripts are included in the references, and any oversights or omissions are the sole responsibility of this author. This work was supported by grants from the National Institutes of Health (GM-41337-02) and the National Science Foundation (DMB-8714352).

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