Synthesis and Application of Circularizable Ligation Probes

BioTechniques 30:584-593 (March 2001)

**Sharyn E. Myer and
Darren J. Day**

Victoria University of Wellington,
Wellington, New Zealand

ABSTRACT

We describe a PCR-based approach for the synthesis of circularizable ligation probes (CLiPs). CLiPs are single-stranded probes that consist of target-specific ends separated by a noncomplementary "linker" sequence. When hybridized to a target, the CLiP forms a nicked circle that may be sealed by DNA ligase only if the 5' and 3' ends show perfect Watson-Crick base pairing, thus enabling the discrimination of single nucleotide polymorphisms. Primers incorporating target sequence at their 5' end and plasmid sequence at the 3' end were used in a PCR amplification. In addition, the antisense primer was 5' labeled with biotin, and the amplification was performed in the presence of fluorescently labeled dUTP. The resulting PCR product was captured with streptavidin-coated paramagnetic beads, and the top strand, which forms the CLiP, was alkali eluted. This PCR-based method has allowed the synthesis of CLiPs that are larger and more highly labeled than has previously been possible, with ligation efficiencies similar to those of the purest chemically synthesized padlock probes. Ligations performed in the presence of cognate or mismatched sequence were analyzed by denaturing PAGE using a fluorescent DNA sequencer. Genotyping using target immobilized to nylon membranes was also performed. The CLiPs were readily able to distinguish between mutant and wild-type alleles for the common genetic disorder, 21-hydroxylase deficiency. Additionally, CLiPs of different lengths were synthesized and compared.

INTRODUCTION

Circularizable ligation probes (CLiPs), also known as padlock probes (1,8–10,13) or C-probes (16), are a new technology that enables the discrimination and detection of single nucleotide polymorphisms (SNPs) (10). These probes consist of two target complementary regions, separated by a "linker" of noncomplementary DNA. When annealed to the target, the 5' and 3' ends of the probe are juxtaposed and form a circle containing a nick (missing phosphodiester bond). The nick can be sealed with a DNA ligase, forming a catenated circle, only if the nick junction shows perfect Watson-Crick base pairing (2,15). A mismatch at or near the junction prevents ligation, thus allowing the discrimination of SNPs.

Nilsson and colleagues (10) pioneered the use of circularizable probes, which they termed padlock probes, because of the nature of the probe to circularize and be locked to its target by catenation when ligated. Allelic variants of human aliphoid repeats have been discriminated by fluorescent in situ hybridization with padlock probes by using metaphase chromosome spreads as targets (9,10). However, detection of single copy gene sequences using CLiPs has so far been unsuccessful (8). Rolling circle replication (RCR) as a means of signal enhancement has been explored and refined (1,6,8,13,16), but discrimination of SNPs in single copy gene sequences on chromosome spreads has remained elusive.

The utility of CLiPs has, in part, been limited to the difficulty in synthesizing large highly labeled CLiPs by conventional phosphoramidite chemistry. Synthesis of long oligonucleotides is problematic because of synthesis fail-

ures at the 3' end, 5' end, or even internally (7). The integrity of the 5' and 3' ends of the CLiPs is essential if they are to function as designed. Alternative oligonucleotide chemistry has improved matters (7), but the utility of CLiPs is still currently limited by an inability to readily synthesize highly labeled probes with defined 5' and 3' ends.

In this work, we describe a PCR-based approach for the synthesis of CLiPs that overcomes these limitations, and we demonstrate their application for the discrimination of mutant and wild-type alleles associated with the common human genetic disorder, 21-hydroxylase deficiency. Ligations using fluorescently labeled CLiPs are shown after analysis by denaturing PAGE using a fluorescent DNA sequencer and also by conventional dot blot analysis of target immobilized to nylon membranes.

MATERIALS AND METHODS

Primers for CLiP Synthesis

Primers were synthesized by Life Technologies (Rockville, MD, USA) or Sigma-Genosys (Castlehill, NSW, Australia). They were designed to discriminate either the deletion allele found in exon 3 or the missense mutation found in exon 4 (leading to the amino acid substitution I172 for N172), from the corresponding wild-type 21-hydroxylase alleles (14). Primer sequences are given in Table 1.

Phosphorylation of Primers

Primers were phosphorylated as previously described (4). Briefly, 1 nmol of primer was incubated for 1 h at 37°C with 10 U T4 polynucleotide kinase

Table 1. Primer Sequences

Name of Primer	Primer Sequence
Exon3wt-139.for	5'-TACTCCCTGCTCTGGAAAGCCCACAAG ACGGATGGCATGACAGT -3'
Exon3wt-352.for	5'-TACTCCCTGCTCTGGAAAGCCCACAAG ACGAGTGGGTTACATCGA 3'
Exon3wt-538.for	5'-TACTCCCTGCTCTGGAAAGCCCACAAGAAT GTATCCGCTCATGAGACAATAAG 3'
Exon3wt-814.for	5'-TACTCCCTGCTCTGGAAAGCCCACAAG GCCCTGATAGACGGTTT -3'
Exon3wt.rev	5' BIOTIN-GTCTCCCAAGGACAGGTCCGGGTAGTTT CGTTGTCAGAAGTAAGTTG 3'
Exon3wt-2338.rev	5' BIOTIN-GTCTCCCAAGGACAGGTCCGGGTAGTTT CACAATCCACACAACATAG 3'
Exon3mut-139.for	5'-TCYCTGCTCTGGAAAGCCCACAAGAAG ACGGATGGCATGACAGT -3'
Exon3mut-352.for	5'-TCYCTGCTCTGGAAAGCCCACAAGAAG ACGAGTGGGTTACATCGA 3'
Exon3mut.rev	5' BIOTIN-CCAA C GACAGGTCCGGGTAGTTTTAGAT CGTTGTCAGAAGTAAGTTG 3'
172.for	5'-CTGTTACCTCACCTTCGGAGAC CGTTTTCCAATGATGAGC 3'
1172.rev	5' BIOTIN-ATGATGCTGCAGGTGAGGAGAG GAATAGTGTATCGGGCGA 3'
N172.rev	5' BIOTIN-TTGATGCTGCAGGTGAGGAGAG GAATAGTGTATCGGGCGA 3'

Nucleotides complementary to pBluescript are shown in bold, and target-specific sequence are shown in normal type. The different sized CLiPs (139, 352, 538, or 814 nucleotides) targeting the exon 3 deletion wild-type allele were all synthesized using a common reverse primer (Exon3wt.rev) and an appropriate forward primer. Likewise, CLiPs targeting the exon 3 mutant allele used the primer Exon3mut.rev. However, the wild-type-specific CLiP of 2383 nucleotides was prepared using the primers Exon3wt-2383.rev and Exon3wt-814.for. CLiPs targeting the exon 4 missense mutation, which results in the amino acid substitution I172 for N172 were synthesized using mutation-specific reverse primers (1172.rev and N172.rev) and a common forward primer (172.for). The mutant sequences targeted in this work arise as a result of recombination between CYP21 and its highly homologous pseudogene CYP21P. There are a number of polymorphisms found within the targeted region that are associated with each gene. These polymorphisms have been incorporated into the primer sequence and are shown underlined.

(Life Technologies) in 70 mM Tris-HCl, pH 7.6, buffer containing 10 mM MgCl₂, 100 mM KCl, 1 mM 2-mercaptoethanol, and 1 mM ATP (Sigma, St. Louis, MO, USA), in a volume of 100 µL. The T4 kinase was inactivated by heating to 95°C for 10 min. The primers were used directly in the PCR.

CLiP Synthesis

CLiPs of different length, specific for normal and mutant, were prepared by PCR amplifying the plasmid Bluescrip[®] using the appropriate forward and reverse primer pairs (Table 1). Each 50-µL PCR amplification contained 0.2 µM each primer and 5 ng linearized pBluescript. The reaction buffer was 20 mM Tris-HCl, pH 8.8, containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton[®] X-100, 20 µM dATP, dGTP, dCTP, 18 µM dTTP, 2 µM fluorescein d-UTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and 1.5 U DEEPVENT[™] exo-

DNA polymerase (New England Biolabs, Beverly, MA, USA).

After an initial denaturation at 95°C for 2 min, amplification was achieved with 20 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s. Amplifications were purified by electrophoresis on a 2% agarose gel, and the required product was excised. The agarose gel slab was solubilized at 55°C in three volumes of 6 M NaI for approximately 10 min, and the product bound to 50 µL 10 mg/mL streptavidin-coated paramagnetic beads (Roche Molecular Biochemicals, Indianapolis, IN, USA), which had previously been washed twice with TNE buffer (10 mM Tris-HCl, pH 8.4, containing 500 mM NaCl and 5 mM EDTA). The biotin-labeled product was allowed to bind for 30 min, and then the beads were washed twice in TNE buffer and once in sterile distilled water. The top strand, which forms the CLiP, was eluted by incubating for 1 min in 50 µL 25 mM NaOH. Product was usually used directly (up

to a maximum of 40% reaction volume) or was desalted into water using a spin column prepared with Sephadex[®] G-50 (Sigma), dried in a vacuum centrifuge, then resuspended in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for later use.

Genotyping of CYP21/CYP21P Alleles: CLiP Ligation with *Tth* Ligase

Plasmid containing CYP21, CYP21P sequence, or PCR amplified 21-hydroxylase product (4), was used as target. A typical ligation contained approximately 20 fmol CLiP and 20–200 ng (20–200 fmol) target in a volume of 20 µL. The reaction buffer was 50 mM Tris-HCl, pH 8.5, containing 10 mM MgCl₂, 50 mM KCl, 10 mM dithiothreitol (DTT), 1 mM NAD⁺, and 80 U *Tth* ligase (2,3). After an initial denaturation of 95°C for 5 min, ligation was achieved by eight cycles of denaturation at 95°C for 30 s and hybridiza-

Research Report

tion/ligation at 55°C for 10 min. Samples were either analyzed on an ABI PRISM™ 377 DNA sequencer running GeneScan® analysis software (both from Applied Biosystems, Foster City, CA, USA) or electrophoresed on a 3% agarose gel and blotted onto nylon membranes. Analysis on an ABI PRISM 377 DNA sequencer used a denaturing (urea) 4% polyacrylamide gel with a 12-cm “well-to-read” distance. Electrophoresis was performed at 750 V for 1–2 h, depending on the expected length of the products. Appropriate size markers were included with each sample. Results were analyzed using GeneScan analysis software version 2.1. In addition, some samples were analyzed by electrophoresis using 3% agarose gels buffered with TAE buffer (40 mM Tris-acetate containing 2 mM EDTA). Samples were loaded in buffer containing 40% formamide and 5% glycerol, subjected to electrophoresis (100 V, 40 min), then alkali transferred onto positively charged nylon membranes (Roche Molecular Biochemicals). Membranes were baked at 120°C for 20 min before blocking in 1% blocking reagent (Roche Molecular Biochemicals) for 30 min. Signal was detected by incubation with a 1:2000 dilution of an anti-fluorescein horseradish peroxidase (HRP)-conjugated antibody (Roche Molecular Biochemicals) for 30 min and chemiluminescence detection (Roche Molecular Biochemicals) as described in the manufacturer’s instructions.

Dot Blots: CLiP Ligation with T4 Ligase

Approximately 20 fmol plasmid, containing either cloned CYP21 or CYP21P sequence, was dotted onto positively charged nylon membranes and allowed to air dry. Membranes were then baked at 100°C for 20 min, boiled in 0.1% SDS for 2 min, and then washed twice in PBS for 10 min. Hybridization was performed in Church buffer (0.5 M Na₂HPO₄, 1% BSA, 1 mM EDTA, pH 8.0, 7% SDS) containing approximately 4 ng (2 µL NaOH eluate) of CLiP at 64°C for 1 h in a volume of 100 µL/cm² membrane. Membranes were then washed twice in PBS for 10 min to remove residual SDS. Lig-

ation of the probes was performed using 3 U T4 DNA ligase (Life Technologies) in 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM MgCl₂, 1 mM ATP, 5 mM DTT, 5% polyethylene glycol-8000 buffer in a volume of 13 µL/cm² membrane. Ligations were then performed for 1 h at 37°C. Membranes were washed once in 2× standard saline citrate (SSC) (30 mM sodium citrate, 300 mM NaCl) containing 0.5% SDS for 20 min and then either washed for 4 min in 0.1% SDS at 94°C (denaturing wash) or 10 min in 0.1% SDS at room temperature (low-stringency wash). Blots were once again washed twice in PBS for 10 min, and the signal was detected by chemiluminescence as before.

Estimation of ss- and dsDNA Concentrations

CLiPs and ds PCR products were quantified by measuring fluorescence enhancement in the presence of an intercalating dye using a fluorescent microplate reader (SPECTRAmax® Gemini; Molecular Devices, Sunnyvale, CA, USA). SYBR® Green I and SYBR

Table 2. Specific Content of Fluorescein in CLiPs of Different Lengths

CLiP Length (Nucleotides)	Fluorophores/Probe
139	2.0
352	5.5
538	8.0
814	12.0
2383	29.0
The specific content was determined by calculating the concentration of the CLiPs and fluorescein content as described in Materials and Methods.	

Green II (Life Technologies) were used as the intercalating dyes with known quantities of a mixture of oligonucleotides or the 1 Kb+ ladder (Life Technologies) as standards when determining ss- and dsDNA quantities.

Measurement of Fluorescein Content of CLiPs

The amount of fluorescein-labeled

Figure 1. Schematic representation of CLiP synthesis. (A) Primers containing a 5' tail (solid black and gray lines) complementary to the target gene are used to PCR amplify a region of pBluescript in the presence of hapten-labeled dNTP. The forward primer is 5' phosphorylated (Phos) and the reverse primer is 5' biotin labeled. The resulting PCR product shown in (B) is labeled with hapten (stars) and incorporates both primers and their complementary sequences (stippled lines). Capture of PCR product with streptavidin-coated paramagnetic beads, followed by NaOH elution of the top strand, releases the CLiP.

Research Report

dUMP incorporated during synthesis was estimated by measuring fluorescence intensity (excitation $\lambda = 490$, emission $\lambda = 530$) using a model LS 50B Luminescence Spectrometer (Applied Biosystems) using fluorescein-dUTPs as standards.

RESULTS AND DISCUSSION

Synthesis of Circularizable Ligation Probes

To overcome the limitations of chemically synthesizing long labeled oligonucleotides for use as CLiPs, we decided to investigate the use of enzymatic methods, in particular primer extension and PCR using thermostable DNA polymerases. The oligonucleotide primers used for these reactions can be readily made by conventional synthetic chemistries. Our approach was to incorporate sequence complementary to that surrounding the target mutation into PCR primers designed to amplify a re-

gion of DNA displaying little homology to the target genome for analysis. To analyze the 21-hydroxylase genes, we designed PCR primers that would amplify a region of the ampicillin gene of pBluescript and incorporated target-specific sequence by including 5' tails that contained a region of either CYP21 or CYP21P sequence (Figure 1). The intervening pBluescript sequence functions as a labeled spacer between the 5' and 3' target-specific ends of the CLiP. Biotin labeling of the reverse PCR primer enables capture of the amplified material with streptavidin-coated paramagnetic beads. Washing followed by alkaline de-

naturation allows recovery of the top strand that forms the CLiP.

The choice of DNA polymerase for amplification was critical, as some polymerases, such as *Taq* DNA polymerase, can add a nontemplate-derived dA to the 3' end of amplification products. Also, many DNA polymerases possess 5' or 3' exonuclease activity, which can degrade the PCR primers or product. Nontemplated addition of nucleotides, or degradation of 5' or 3' termini, will prevent the CLiP from functioning as designed. Another consideration addressed was the efficiency of incorporation with hapten-modified dNTPs. Fluorescein-mod-

Figure 2. Analysis of ligation products using GeneScan analysis software. CLiPs were designed to target both the exon 3 wild-type and mutant (deletion) allele of CYP21. Wild-type CLiP was labeled with fluorescein (gray), and mutant CLiP was labeled with rhodamine (black). Ligations were performed as described in Materials and Methods, and the ligation products were separated using a 4% denaturing polyacrylamide gel and analyzed using GeneScan analysis software. Panel A shows that in the presence of wild-type target, only the wild-type-specific CLiP (gray) is circularized. Similarly, in the presence of mutant target (panel B), only the mutant specific CLiP (black) is circularized. In the absence of target (panel C), neither probe is circularized. As expected, dephosphorylation of the CLiP prevented its ligation (panel D), and the circularized CLiP was resistant to exonuclease digestion (panel E). Exclusively wild-type CLiP (gray) and target were used in the ligation reactions shown in panels D and E.

Research Report

ified dNTPs are incorporated well by most thermostable DNA polymerases used in PCR; however, rhodamine and digoxigenin-labeled nucleotides are poorly incorporated by some polymerases. Amplifications for CLiP synthesis were performed with DEEPVENT exo⁻ because this enzyme has no 5' or 3' exonuclease activity, has minimal terminal transferase activity, and incorporates hapten-modified dNTPs well. Using this enzyme and the outlined approach, we were able to synthesize CLiPs of much greater length with a higher specific incorporation of hapten than has previously been possible.

Application of Circularizable Ligation Probes

Ligations were performed using the thermostable DNA ligase, *Tth* ligase (2,3), and CLiPs that were 139 nucleotides in length and labeled with fluorescein (wild-type allele) or with rhodamine (mutant allele). Both CLiPs were included in the ligation that con-

tained either wild-type or mutant target. The ligation products were analyzed by denaturing PAGE on a ABI PRISM377 DNA sequencer running GeneScan analysis software. Circular product was detected by its decreased mobility compared to the linear CLiP when analyzed by denaturing PAGE. In the presence of wild-type target, a fluorescein-labeled circular product was formed (Figure 2A). Conversely, when mutant sequence was used as target, only a product from the mutant-specific CLiP was formed (Figure 2B). As expected, neither CLiP circularized in the absence of target (Figure 2C) nor when dephosphorylated (Figure 2D). Additionally, circular ligation product was resistant to exonuclease digestion (λ exonuclease or exonuclease III), while linear CLiP was not (Figure 2E). This is consistent with other reports that circular oligonucleotides are resistant to digestion because they have no free 5' or 3' ends (5,10,11,16). The data indicate that the CLiPs show allelic discrimination and are only circularized in the

presence of cognate target sequence.

Genotyping of two individuals with known mutations in CYP21 was performed using CLiPs (Figure 3). The CLiPs were readily able to identify that one subject was heterozygous for the eight base pair deletion allele in exon 3 (top panel), and the other was homozygous for the point mutation (T to A) in exon 4 that results in the amino acid substitution, I172 for N172. The genotyping was concordant with those previously obtained for these individuals.

Additional ligations were performed using *Tth* ligase and a 352-nucleotide CLiP, and the ligation products were analyzed by agarose gel electrophoresis, followed by Southern transfer and chemiluminescent detection, as described in Materials and Methods. The fluorescein-labeled CLiPs were subjected to thermal cycling with either wild-type or mutant sequence as target. Figure 4 shows that excess linear CLiP was detected in all samples but circular CLiP, and CLiP catenated to the target was only formed in the presence of per-

Figure 3. Genotyping two patients with mutations in CYP21 using CLiPs. Ligations were performed as described in Materials and Methods, separated using a 4% denaturing polyacrylamide gel, and analyzed using GeneScan analysis software. CLiPs of 352 nucleotides specific for the wild-type (gray) and deletion (black) were used to differentiate between the deletion alleles in exon 3 of CYP21. The top panel shows that the individual is heterozygous for this mutation, as circular product for both wild-type and mutant CLiPs were formed. Similarly, CLiPs of 145 nucleotides in length were designed to target the missense mutation in exon 4 (T to A), which results in isoleucine 172 being converted to an asparagine residue. The wild-type (I172) and mutant (N172) CLiPs are shown in gray and black, respectively. The individual genotyped homozygous for the N172 allele, as only mutant-specific probe was circularized.

Figure 4. Analysis of CLiP ligations on agarose gels. Ligations were performed as described in Materials and Methods, using a 352-nucleotide exon 3 wild-type-specific CLiP labeled with fluorescein and plasmid clones containing either mutant or wild-type sequences as target. Ligation products were separated by electrophoresis on a 3% agarose gel, blotted onto nylon membranes, and detected with an anti-fluorescein HRP-conjugated antibody and chemiluminescent detection. Lane 1 contained wild-type target; lane 2, mutant target; lane 3, no target; and lane L, molecular weight size markers (1 Kb+ ladder labeled with fluorescein). Free CLiP was detected in lanes 1–3, but only circular CLiP and CLiP catenated to target were formed in the presence of cognate (wild-type) sequence.

Research Report

fectly matched (wild-type) target. Both free circular CLiP and CLiP locked to the target were detected when ligation products were analyzed by agarose gel electrophoresis. Free circular CLiP was

formed as thermal denaturation allowed the circularized CLiP to diffuse along the length of the target molecule, such that it may slip off a free end if a linear target is used (10).

Figure 5. Ligation of CLiPs to target immobilized on nylon membranes (dot blots). Each membrane contained immobilized cloned wild-type target (top left), mutant target (top right), and plasmid containing no target (bottom left). The membranes were hybridized with CLiP targeting wild-type sequence (A, B, and C) or CLiP targeting mutant sequence (D, E, and F). Ligations were performed using T4 DNA ligase; however, ligase was omitted from membranes C and F, as described in Materials and Methods. The membranes were then either subjected to a low-stringency wash (0.1% SDS at room temperature, membranes A and D) or a denaturing wash (0.1% SDS at 94°C, membranes B, C, E, and F). CLiPs annealed to all targets, and were not removed by a low-stringency wash (A and D). However, only CLiPs that were catenated to their cognate sequences remained bound after the highly denaturing wash (B and E). Ligation was essential for catenation of the CLiPs to their target (C and F).

Figure 6. Comparison of signal intensity using CLiPs of different lengths. Ligations were performed with 3 fmol each probe and excess target (15 fmol) using 80 U *Tth* ligase and 10 thermal cycles of denaturation at 95°C for 30 s and annealing/ligation at 55°C for 10 min. The signal intensity was determined by integration of the peak area corresponding to circular product. Values are expressed as a percentage of the highest signal obtained.

In addition to genotyping aqueous DNA samples, we wished to investigate the use of CLiPs with target immobilized on nylon membranes. Accordingly, target was dotted onto nylon membranes and then hybridized and ligated with CLiP specific either for wild-type (Figure 5, A, B, and C) or mutant target sequence (Figure 5, D, E, and F). When washed at room temperature (low-stringency wash), unligated CLiP remained hybridized to all targets (Figure 4, A and D). Washing under strongly denaturing conditions (94°C) showed that the wild-type CLiP was only locked onto its cognate target (Figure 5B). Figure 5D shows the mutant-specific CLiP was also specific for its cognate sequence. When DNA ligase was omitted from the reaction, the CLiPs hybridized to target but were not topologically locked; hence, they were readily dissociated by the 94°C denaturing wash (Figure 5, C and F).

The experiments with immobilized target used T4 DNA ligase for the ligation of CLiPs and the discrimination of target. Similar experiments using *Tth* ligase surprisingly failed to ligate the CLiPs to immobilized target. We have also noted difficulty when using *Tth* ligase to ligate adjacently hybridized oligonucleotides in the ligation detection reaction (LDR) assay of Day et al. (4) when the target strand is immobilized to magnetic beads (results not shown). It is possible that the T4 and *Tth* ligases have different steric requirements for ligation of probes with solid phase templates.

To investigate if there was an upper limit to the size of CLiP that can be synthesized by our PCR-based method, we designed primers to make additional CLiPs of 538, 814, and 2383-nucleotides. All of these probes were shown to ligate with high specificity; however, the maximum yield of ligated product obtained differed. Up to 84% of the 139-, 352-, 538-, and 814-nucleotide CLiPs could be ligated, but the largest CLiP (2383 nucleotides) was unable to be ligated to yields greater than 30%. The less than 100% yield (for the four smaller CLiPs) is probably due in part to a combination of primer synthesis failure or polymerase modifications of the primers resulting in mismatches or gaps near the nick junction, as well as incomplete phosphorylation of CLiP.

Steric factors are likely to have contributed to the low yield obtained with the largest CLiP. While all the CLiPs other than the 2383-nucleotide CLiP could be ligated to high yields, the smaller required fewer cycles and less *Tth* ligase than the larger probes. Table 2 shows the specific content (fluorophores/probe molecule) of each of the CLiPs; as expected, the number of incorporated fluorophores increased proportionately with size. The number of fluorophores introduced varied from 2 for the 139-nucleotide CLiP to 29 for the 2383-nucleotide CLiP. For comparison, the chemically synthesized probes used to differentiate the α satellite repeats in human metaphase spreads used by Nilsson et al. (9), contained two haptens/probe molecule. The specific content of the CLiPs described in this work could be further increased by increasing the ratio of labeled dNTP to nonlabeled dNTP during the PCR step. We used a ratio of 1:10 to ensure efficient PCR amplification, but ratios of 1:4 have been used in other PCR labeling methods (12). Experiments in which identical molar amounts of the different CLiPs were ligated with excess target, greater fluorescence signal was obtained with increasing probe length, with the exception of the 2383-nucleotide CLiP (Figure 6). The lower signal intensity with this CLiP is because of the lower yield of ligated product obtained, as previously discussed.

Our enzymatic approach for synthesizing CLiPs offers several advantages over previous methods in that it has allowed us to readily make circularizable probes that are much longer, with greater incorporation of hapten than has been previously been possible. As the probes are synthesized by PCR, they can be economically and rapidly produced in large quantities. Additionally, these probes may be ligated to high yield with no need for purification steps to remove molecules with imperfect 5' or 3' termini. This is possible because the synthesized oligonucleotides used to make the CLiPs are short, and we have used an optimal DNA polymerase for the enzymatic synthesis. One of the advantages of incorporating large linkers into the CLiPs, other than increasing the amount of hapten incorporated, is that the content of the linker region can be made

functional. We are currently investigating the use of incorporating functional sequence elements into the spacer region to increase the utility of the CLiPs.

ACKNOWLEDGMENTS

We thank the Wellington Medical Research Foundation for financial support and Francis Barany for the kind gift of the *Tth* DNA ligase.

REFERENCES

1. Banér, J., M. Nilsson, M. Mendel-Hartvig, and U. Landegren. 1998. Signal amplification of padlock probes by rolling circle replication. *Nucleic Acids Res.* 26:5073-5078.
2. Barany, F. 1991. Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc. Natl. Acad. Sci. USA.* 88:189-193.
3. Barany, F. and D.H. Gelfand. 1991. Cloning, overexpression and nucleotide sequence of a thermostable DNA ligase-encoding gene. *Gene* 109:1-11.
4. Day, D.J., P.W. Speiser, P.C. White, and F. Barany. 1995. Detection of steroid 21-hydroxylase alleles using gene-specific PCR and a multiplexed ligation detection reaction. *Genomics* 29:152-162.
5. Dolinnaya, N.G., M. Blumenfeld, I.N. Mendenkova, T.S. Oretskaya, N.F. Krynetskaya, M.G. Ivanovskaya, M. Vasseur, and Z.A. Shaborova. 1993. Oligonucleotide circularization by template-directed chemical ligation. *Nucleic Acids Res.* 21:5403-5407.
6. Fire, A. and S. Xu. 1995. Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. USA.* 92:4641-4645.
7. Kwiatkowski, M., M. Nilsson, and U. Landegren. 1996. Synthesis of full-length oligonucleotides: cleavage of apurinic molecules on a novel support. *Nucleic Acids Res.* 24:4632-4638.
8. Lizardi, P.M., X. Huang, Z. Zhu, P. Bray-Ward, D.C. Thomas, and D.C. Ward. 1998. Mutation detection and single-molecule counting using isothermal rolling-circle amplification. *Nat. Genet.* 19:225-232.
9. Nilsson, M., K. Krejci, J. Koch, M. Kwiatkowski, P. Gustavsson, and U. Landegren. 1997. Padlock probes reveal single-nucleotide differences, parent of origin and *in situ* distribution of centromeric sequences in human chromosomes 13 and 21. *Nat. Genet.* 16:252-255.
10. Nilsson, M., H. Malmgren, M. Samiotaki, M. Kwiatkowski, B.P. Chowdhary, and U. Landegren. 1994. Padlock probes: circularizing oligonucleotides for localized DNA detection. *Science* 265:2085-2088.
11. Prakash, G. and E.T. Kool. 1992. Structural effects in the recognition of DNA by circular oligonucleotides. *J. Am. Chem. Soc.* 114:3523-3527.
12. Steffen, C. 1999. Roche Molecular Biochemicals PCR Applications Manual 2nd edition.

Roche Diagnostics GmbH, Mannheim, Germany.

13. Thomas, D.C., G.A. Nardone, and S.K. Randall. 1999. Amplification of padlock probes for DNA diagnostics by cascade rolling circle amplification or the polymerase chain reaction. *Arch. Pathol. Lab. Med.* 123:1170-1176.
14. White, P.C., M.T. Tusie-Luna, M.I. New, and P.W. Speiser. 1994. Mutations in steroid 21-hydroxylase (CYP21). *Hum. Mutat.* 3:373-378.
15. Wu, D.Y. and R.B. Wallace. 1989. Specificity of the nick-closing activity of bacteriophage T4 DNA ligase. *Gene* 76:245-254.
16. Zhang, D.Y., M. Brandwein, T.C.H. Hsuih, and H. Li. 1998. Amplification of target-specific, ligation-dependent circular probe. *Gene* 211:277-285.

Received 16 March 2000; accepted 20 September 2000.

Address correspondence to:

Dr. Darren Day
School of Biological Sciences
Victoria University of Wellington
PO Box 600
Wellington, New Zealand
e-mail: darren.day@vuw.ac.nz