Targeted gene walking polymerase chain reaction

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ABSTRACT

We describe a modification of a polymerase chain reaction method called 'targeted gene walking' that can be used for the amplification of unknown DNA sequences adjacent to a short stretch of known sequence by using the combination of a single, targeted sequence specific PCR primer with a second, nonspecific 'walking' primer. This technique can replace conventional cloning and screening methods with a single step PCR protocol to greatly expedite the isolation of sequences either upstream or downstream from a known sequence. A number of potential applications are discussed, including its utility as an alternative to cloning and screening for new genes or cDNAs, as a method for searching for polymorphic sites, restriction endonuclease or regulatory regions, and its adaptation to rapidly sequence DNA of lengthy unknown regions that are contiguous to known genes.

INTRODUCTION

The polymerase chain reaction is a technique that has revolutionized molecular biology by virtue of its utility in amplifying microgram amounts of a specific, targeted gene from as little as a single copy of starting template DNA (1–4). One of the limitations of this technology, however, is the need for the sequence of two target specific primers that flank the region one wishes to amplify. This places a limitation on the technology; namely, that regions that lie outside the boundaries of known sequences cannot be amplified without major modifications in the technology that include steps such as ligation and circularization ('inverse' PCR), the ligation of adaptors to the ends of the amplified sequence, or tailing of specific deoxynucleotides to produce a site to which a primer can hybridize (5–8). In each of these techniques, additional steps must be taken to artificially construct a region for which the primer sequence is known, that brackets the unknown sequence. Each of these techniques requires further steps that may decrease the efficiency of recovery of the product, increase the chances of contamination, and are laborious. We describe a modification of the polymerase chain reaction that allows the amplification of unknown sequences adjacent to a known sequence without intervening steps. The method is based upon our observation that

a primer may initiate PCR at either unknown or specific target sequences which bear only partial homology to the 3' end. The technique can be used to 'walk' along the DNA sequence or to search for nucleotide sequences that have been designed by the user. The method allows the production of micrograms of DNA of unknown sequence that occur upstream or downstream from a single region of targeted DNA.

METHODS

Primers and template DNAs

Three categories of PCR primers have been used in these studies: 'targeted' primers that hybridize to a specific, target sequence [e.g. human Kirsten ras protooncogene exon 2 (Kras, ref. 9) and mitochondrial D-loop (mtD, ref. 10) in the studies described herein]; 'internal' detection primers for use in the oligomer extension assay, which are 'targeted' primers that are located a short distance internally with respect to the PCR primers, and 'walking' primers that have been used to hybridize to sequences that are not known to preexist either upstream or downstream from the target gene (Table 1 and Fig. 1). For the experiments described, primers that were previously synthesized in our laboratory for the purpose of amplifying genes other than Kras and mtD were used as random 'walking' primers. The oligonucleotides were synthesized in our laboratory using the phosphoramidite method on a Cruachem synthesizer (Cruachem Corp., Herndon, VA).

The template DNA used in the Kras 'gene walking' experiments was obtained after precipitating total nucleic acids from 1×10^8 HeLa cells by standard techniques (11). The template DNA fragment used in the mtD experiments was obtained by amplifying a 0.75 kilobase pair fragment from HeLa cell DNA encompassing nucleotides 16441–16569 and 0–617 (10) of the human mtDNA sequence with primers specific for mammalian D-loop sequences [mtD-1 and mtD-3, Table 1] in a total volume of 50 μ l using 50 ng of each primer and PCR conditions detailed below. The 0.75 kb band was excised from a 2% (w/v) ethidium bromide stained agarose gel, eluted with a Gelex extractor (Genex Corp., Gaithersburg, MD), the DNA was precipitated with 0.1 vol of 3 mol/L sodium acetate (pH 5.2), resuspended in 20 μ l of water, and the concentration was determined spectrophotometrically.

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Gene Walking PCR procedure

The overall protocol for 'gene-walking PCR' consists of three consecutive steps:

1) A series of small volume (e.g. 25 μ l) PCR reactions are performed in parallel, with identical components in each tube (including the 'targeted' primer) except for different 'walking' primers.

2) The oligomer-extension assay is performed on a 10 μ l aliquot of each PCR reaction with a nested, kinased internal primer (12). This procedure allow identification of products that represent 'walks' from the targeted site.

3) The labeled band is excised from the gel, re-amplified, and directly sequenced. The specific conditions for the 'gene-walking' PCR experiments described in this report are as follows:

Fifty ng of total cellular DNA or 50 pg of the purified 0.75 kb amplified fragment was used as a template in 25 μ l of buffer containing 80 mM Tris-HCl (pH 9.0), 20 mM ammonium sulfate, 10 mM MgCl₂, 100 μ M each dATP, dCTP, dGTP, and dTTP, 1 ng/ μ l (2.5 pmol/25 μ l reaction) of the 'targeted' oligonucleotide primer, 2 ng/ μ l (5 pmol/25 μ l reaction) of the 'walking' primer, and 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). The mixture was overlaid with 1 drop of mineral oil and in cases in which cellular DNA was the template, underwent 30 cycles of PCR with denaturation at 98°C for 20 sec, annealing at 50°C for 30 seconds, and extension at 72°C for 90 sec. For PCR reactions using the purified 0.75 kb mtDNA fragment as a template, the mixture underwent 30 cycles of amplification at 98°C for 20 sec, annealing at 40°C for 30 sec, and polymerization at 72°C for 30 sec.

Oligomer extension assay

Identification of targeted PCR products was performed with the oligomer-extension 'hot blot' assay as previously described (12). Ten μ l aliquots of the PCR reaction were mixed with 1 ng of kinased, 'internal' detection primer. The mixture underwent a single cycle at 98°C for 20 sec, 50°C for 30 sec, and 72°C for one minute, and the contents were electrophoresed on 2% (w/v) agarose, placed onto a piece of Nytran membrane (Schleicher and Schuell, Kenne, NH) dried for 45 minutes under vacuum at 80°C using a gel dryer, and placed into an X-ray cassette with two intensifying screens and exposed to X-ray film for 3 hrs. at -70°C.

Figure 1. Schematic diagram of targeted gene walking PCR. In the diagram shown, the 'targeted' and 'internal' primers are complementary to a known sequence, and the 'walking' primers are complementary to stretches of DNA downstream from the 'targeted' and 'internal' primers. Stretches of unknown sequence that are located upstream from the known sequence can be amplified by synthesizing the complementary strands of the 'targeted' and 'internal' primers and reversing their order.

Secondary amplification of detected, target specific PCR products

Bands shown to be positive for the target sequence by the oligomer extension assay were either excised from the dried agarose gel or excised from a duplicate gel where the correct band could be visually identified from the background of non-target specific PCR products on an ethidium bromide stained gel. The band was eluted by incubating the gel slice with 50 μ l of water in a 1.7 μ l microfuge tube and shaking in a rotary shaker (Lab-line Orbit Environ-shaker, Lab-Line Instruments, Inc., Melrose Park, Ill.) for three hours. A 5 μ l aliquot of the eluted DNA was used directly as a template in a 100 μ l secondary asymmetric PCR reaction using the same 'targeted' primer (100 pg/ μ l or 1 pmol/100 μ l) in the presence of twenty-fold excess of 'walking' primer (2 ng/ μ l or 20pmol/100 μ l). The PCR product was desalted by Sephadex G-25 (Pharmacia LKB, Piscataway, NJ) spin-column chromatography and sequenced with either the 'targeted' primer or an internal detection primer using a Sequenase kit per manufacturer's recommendations (United States Biochemical Corp., Cleveland, OH).

RESULTS

Overall strategy of 'targeted gene walking' PCR

The 'gene walking' procedure is a modification of PCR that allows the direct, targeted amplification of unique stretches of contiguous DNA without a priori knowledge of sequence information beyond the targeted priming site. This is accomplished by using a single targeted amplification primer that anneals to the known sequence, and a second 'walking' primer

Figure 2. Products of targeted gene walking PCR of mtDNA fragment. The 0.75 kb amplified fragment of the human mtDNA D-loop was used as a template in individual PCR reactions with mtD-1 as the 'targeted' primer, and an array of 'walking' primers, and mtD-2 as the 'internal' detection primer for the oligomer-extension assay. Aliquots of the 30 cycle PCR reaction underwent oligomer-extension as described in Methods with a kinased mtD-2 primer, were electrophoresed on a 2% (w/v) agarose gel in the presence of ethidium bromide, and photographed with UV transillumination (Panel A) prior to drying and autoradiography (Panel B). The following 'walking' primers were used in the PCR reactions: Lane a=W-1, b=W-2, c=W-3, d=W-4, e=W-5, f=Kras-2, g=W-6, h=W-7, i=W-8, j=W-9, k=W-10, l=W-11, m=W-12, n=W-13, o=W-14, p=W-15, q=W-16. Lane r=Hind III digested lambda DNA marker. The size of the fragment in base pairs is indicated to the right of the photograph.

that has sequences that are not known to be complementary to a particular region of contiguous DNA near the target sequence. Amplification products are produced when the 'walking' primer hybridizes with sufficient base-pairing to serve as a suitable substrate for Taq-mediated polymerization. Targeted amplification can occur provided the 'walking' primer is extended toward the 'targeted' primer, and is within a 5 kb size range. The overall strategy is diagrammed in Fig. 1. The 'targeted' primer is an oligonucleotide that is exactly complementary to a known sequence, and is directed toward the unknown sequence. The 'internal' detection primer is designed so that there is no overlap between it and the 'targeted' primer, yet still hybridizes to the same strand of the known sequence. 'Walking' primers will hybridize to multiple sites on both the coding and complementary strands of template DNA. Targeted PCR products, however, will only be produced when a 'walking' primer anneals to a DNA strand that is contiguous with and complementary to the strand of DNA to which the 'targeted' primer has hybridized. In the simplest case, a single PCR reaction is performed with a 'targeted' primer and a single 'walking' primer, and the products are analyzed by oligomer-extension with a kinased 'internal' primer.

'Gene-walking' of a mtDNA fragment

The method is demonstrated using a 0.75 kb fragment of DNA that has been amplified from the human mitochondrial D-loop

and subsequently purified by extraction from an agarose gel (Fig. 2). The 'targeted' primer in this example is the same primer that was used as the upstream amplification primer in the original PCR reaction that generated the 0.75 kb fragment (primer mtD-1, Table 1). The 'walking' primers are oligonucleotides that had been previously synthesized in our laboratory for targeted amplification of nuclear genes unrelated to the mitochondrial D-loop, and serve as a set of primers with essentially arbitrary sequences (Table 1). Even with this purified template, a large number of products from less than 100 bp to greater than 500 bp are visible upon ethidium bromide staining of a 2% agarose gel after amplification with the combination of mtD-1 and each of these individual primers (Panel 1A). Less than 50% of the bands that are visible by ethidium bromide staining, however, are amplification products that extend downstream from the mtD-1 annealing site into adjacent regions, as demonstrated by the oligomer-extension assay (12) using the kinased internal

Table 1. Oligonucleotide Primers used in PCR and Oligomer-Extension Assays

Primer	Sequence
Targeted PCR and Oligomer-Extension Primers:	
mtD-1	5'-CTCTT/CCTCGCTCCGGGCCAT-3'
mtD-2	5'-CTTAAATAAGACATCA/TCGATG-3'
mtD-3	5'-CATTTTCAGTGC/TC/TTTGCTTT-3'
Kras-1	5'-AAGGATCCGCTGCTGAAAATGAC-3'
Kras-2	5'-ACTCTTGCTACGCCACC-3'
Kras-3	5'-ACTCATGAAAATGGTCAG-3'
Walking Primers:	
W-1	5'-ACTACTA/C/TGGGTATCA/G/TAATCCT-3'
W-2	5'-AAAA/GAA/TTGGTGGCAACAATACC-3'
W-3	5'-AAAGTCGTTTAGACTATAAA-3'
W-4	5'-AAATTTAAGCTTCTAGAAATCC-3'
W-5	5'-CATTATGATTTGTCTTAA-3'
W-6	5'-ACGTGGCCACGTAGGCCAAAAAAAAAAAA-3'
W-7	5'-TTTAAGCTTCTAGAAATCCCCCCCC-3'
W-8	5'-AAGGCTGGGACCAACCT-3'
W-9	5'-GTGGCATCGAGAAAGCTGTC-3'
W-10	5'-AGAAGTTTCGATGGAAGCTC-3'
W-11	5'-AATTACCGCAACGGCTGGCAT-3'
W-12	5'-GTCAATTGTTAT/GTATTCATAT-3'
W-13	5'-AAGCTCAGATCTACCTGCCTGAGG-3'
W-14	5'-CCACGATGCGTCCGGCGT-3'
W-15	5'-TTCATTTATAATCCTTATCA-3'
W-16	5'-TCACAACACGAGCTGACG-3'
W-17	5'-ACGTGCGGCCGCTTTTTTTTTTTTT-3'
W-18	5'-CTGATGCAAATAGTTGGTGGG-3'
W-19	5'-TGCTCGTTACCCACCAAGCC-3'
W-20	5'-AAGAATTCATACAGGGTCTTCAT-3'
W-21	TACAAAGACAAACAGTATTACAACATAGTTTT-3'
W-22	5'-GTGAGAAACACACCACAA-3'
W-23	5'-AAAGCAA/GA/GCACTGAAAATG-3'

'Targeted' amplification primers for the human mtDNA D-loop region consist of mtD-1 (upstream) and mtD-3 (downstream). mtD-2 is an 'internal' detection primer for the oligomer-extension assay. The 'targeted' amplification primers for the c-Ki-ras protooncogene consist of Kras-1 (upstream) and Kras-3 (downstream); Kras-2 is the internally nested oligomer-extension primer.

Figure 3. Sequencing autoradiograms of products of targeted gene walking PCR of 0.75 kb mtDNA fragment. Four bands were excised from the gene walking experiment shown in Fig. 2 (A=Lane 0, B=Lane J, C=Lane H, and D=Lane K), and sequenced with primer mtD-2. The sequencing autoradiogram reveals the region of the mtDNA sequence to which each of the 'walking' primers has annealed during the PCR reaction. The sequences of the PCR products produced with mtD-1 and primers W-14 (A), W-9 (B), W-7 (C) and W-10 (D) are read as follows, where the nucleotides represented by capital letters correspond to the amplified mtDNA sequence, and those in small letters correspond to the complementary strand 'walking' primer sequence (annealing site of primer): A=5'-ACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTGGGGGGTGTGCACGCGACAGCTTGCAGAGcgcggcagc-3' B=5'-ACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTGGGGGGTGTGCACGCGacagcttctcg-3' C=5'-ACTCACGGGAGCTCTCCATGCATTTGGTATTTTCGTCTgggggggggaattctag-3' D=5'-TCACGGagcttccatcgaaa-3'

Table 2. 'Walking' primer hybridization sites within the human mtDNA fragment

Product	DNA Sequence:	
Lane B	primer W-2	3'-CCATA ACAAC GGGTG GTA/TAA/G AAA-5'
	mtDNA	5'-GGTAT TTTCG TCTGG GGGGT ATG-3'
Lane D	primer W-4	3'-CCCTT AAGAT CTTCG AATTT AAA-5'
	mtDNA	5'-GGGAG CTCTC CATGC ATTG GTA-3'
Lane H	primer W-7	3'-CCCCC CCCCC CTAA GATCT TCGAA TTT-5'
	mtDNA	5'-GGGGG GTGTG CACGC GATAG CATTG CGA-3'
Lane J	primer W-9	3'-CTGTC GAAAG AGCTA CGGTG-5'
	mtDNA	5'-GATAG CATTG CGAGA CGCTG-3'
Lane K	primer W-10	3'-CTCGA AGGTA GCTTT GAAGA-5'
	mtDNA	5'-GAGCT CTCCA TGCAT TTGGT-3'
Lane L1	primer W-11	3'-TACGG TCGGC AACGC CATT A-5'
	mtDNA	5'-ATTCC TGCCT CATCC TATTA T-3'
Lane L2	primer W-11	3'-TACGG TCGGC AACGC CATT A-5'
	mtDNA	5'-ATGTC GCAGT ATCTG TCTTT G-3'
Lane M	primer W-12	3'-TATAC TTATT/G ATTGT TAACT G-5'
	mtDNA	5'-ATAAT AATAA CAATT GAATG T-3'
Lane O	primer W-14	3'-TGCGG CGTGC GTAGC ACC-5'
	mtDNA	5'-ACGCT GGAGC CGGAG CAC-3'
Lane Q	primer W-16	3'-GCAGT CGAGC ACAAC ACT-5'
	mtDNA	5'-CGTCT GGGGG GTATG CAC-3'

Sequence of the 'walking' primer and corresponding mtDNA hybridization site in ten representative 'gene-walking' PCR products that were positive by the oligomer-extension assay. All products were excised from the gel shown in Fig. 2 and reamplified as described in Methods prior to sequencing. Lane L contained two products, both of which were excised, reamplified, and sequenced.

extension primer mtD-2 [Panel 1B]. The size of these products represents the distance from mtD-1 to the initial permissive annealing site of the 'walking' primer. The remaining products that do not hybridize to the internal primer represent non-targeted amplification products that are produced by the 'walking' primer alone, or in combination with mtD-1.

Analysis of primer annealing sites

The bands identified by the oligomer-extension assay as positive for sequences adjacent to mtD-1 were excised from the dried gel and reamplified using the mtD-1 and the same 'walking' primer used in the initial PCR reaction. These products were then directly sequenced with the internal detection primer mtD-2. The sequencing autoradiograms on a representative selection of these products demonstrate the annealing sites of the 'walking' primer within the mitochondrial DNA sequence (Figure 3). An analysis of these sequences demonstrates the diversity of annealing sites found by the 'walking' primers within the mitochondrial DNA sequence (Table 2); all contain mismatches with respect to the sequence of the latter half of the 'walking' primer.

An analysis of the frequency of complementary base pairs in the 'walking' primer hybridization sites as a function of distance from the 3' end is shown in Fig. 4. Primer-template annealing sites from 22 different oligomer-extension positive bands were analyzed. Mismatches were not seen at the last 2 nucleotide positions at the 3' end of the 'walking' primers; however, more proximally, complementarity appears to decline exponentially. Throughout 20 nucleotides of primer sequence, there are an average of 9 complementary base pairs, which represents only 4 base pairs in excess of that expected by random chance. Amplification products were seen with as many as 60% mismatched nucleotides within the 'walking' primer relative to the DNA template.

Previous investigators have emphasized the importance of annealing temperature as a variable that affect the stringency of the primer-template interaction (1,3). We have observed that

Figure 4. Plot of nucleotide distance from 3' end of the 'walking' primer vs. the percentage of nucleotides at that distance which exhibit complementary base-pairing. The solid line shows the best least squares fit to a model of exponential reduction in the requirement for homology, assuming a plateau value of 25% homology that is expected on a random basis.

increasing the annealing temperature from 40°C to 60°C produces a decrease in both specific and non-specific amplification products, and as anticipated, 'walking' primers with higher homology to the target sequence and with higher G-C content were more stable at increased annealing temperatures (data not shown).

'Gene-walking' on genomic DNA template

To demonstrate the general utility of this procedure for amplification of contiguous stretches of DNA from a complex template, the 'gene-walking' method was performed to target the first exon of the protooncogene c-Ki-ras 2 using HeLa cell genomic DNA as a template in the PCR reaction (Fig. 5). The 'targeted' primer is located at the intron-exon boundary and is directed downstream. After PCR amplification with an array of 'walking' primers (Fig. 5A), oligomer-extension assays were performed using an internal detection primer located at the latter half of the first exon in order to identify products that contain sequences contiguous to the 'target' primer (Fig. 5B). These products were then excised from the gel, reamplified, and directly sequenced with Kras-1 to verify the identity of the amplification product as a contiguous stretch of DNA adjacent to the Kras first exon (data not shown). In this case, products that range in size from less than 100 bp up to 2 kb pairs are visible on the ethidium bromide stained agarose gel; however, only two 'gene-walking' products of approximately 500 bp (Lane e) and 100 bp (Lane h) are clearly visible in the oligomer-extension autoradiogram. The positive control, in which two Kras 'targeted' amplification primers were used in the PCR reaction is also visible as a single band of 218 bp on both the agarose gel and the oligomer-extension autoradiogram (Lane l).

DISCUSSION

We have described a general method for the amplification of contiguous stretches of DNA of unknown sequence from a targeted, specific site for which some sequence information is available. In contrast to previously published methods for isolating such sequences, there are no intervening steps such as ligation, cloning into plasmids or phage vectors, and no need for screening of libraries to isolate the desired sequence. The method as described, in conjunction with the oligomer-extension assay, allows the rapid detection and isolation of stretches of DNA either upstream or downstream from a known sequence. We have used this technique to isolate and directly sequence over 1 kilobase of mitochondrial DNA from the Atlantic salmon (*Salmo salar*), for which published sequence information does not exist (manuscript in preparation).

A variety of PCR applications and modifications have been developed which rely on the assumption that near perfect complementary primer-template pairing is occurring during the amplification reaction. Examples include strategies such as substitution of the 3' nucleotide for the detection of mutations (13), substitution of nucleotides proximal to the 3' end for the construction of restriction sites used for detection of mutations (14), or during amplification of polymorphisms for DNA fingerprinting purposes (15,16). Our results demonstrate, however, that oligonucleotide primers containing large numbers (50%) of mismatches can serve as amplification primers, as long as there is partial homology at the 3' end of the primer and correct base pairing of the last two nucleotides at the 3' end. This non-stringent primer template interaction provides the basis for the production of the multiple bands that do not hybridize to the 'internal' primer, and presumably represent regions that contain DNA sequences with partial homology to the 'targeted' or the 'walking' primers. In particular, our results suggest that caution should be exercised in the interpretation of bands produced from 'random' primed PCR reactions such as those used for DNA

Figure 5. Products of targeted gene walking PCR of c-Ki-ras 2. HeLa DNA was used as a template in individual PCR reactions with Kras-1 as the 'targeted' primer, an array of 'walking' primers, and Kras-2 as the 'internal' detection primer for the oligomer-extension assay. Aliquots of oligomer-extension were electrophoresed on 2% (w/v) agarose in the presence of ethidium bromide, photographed with UV transillumination (Panel A), dried and autoradiographed (Panel B). 'Walking' primers are as follows: Lane a=W-6, b=W-17, c=W-18, d=W-19, e=W-2, f=W-14, g=W-4, h=W-20, i=W-21, j=W-22, k=W-23. Lane l contains a 218 bp PCR product from Kras-1 and Kras-3, which serves as a positive control for the oligomer-extension assay. Lane m=Hind III digested lambda DNA.

fingerprinting purposes (15,16). We have observed that minor alterations in the PCR parameters and template DNA quality can produce dramatic differences in the pattern of bands produced by PCR when using primers that are not perfectly complementary to the target template sequence. Apparent loss of bands or shifts in the position of bands can occur due to effects of non-perfect base pairing between the random primer and the template that results in the elimination or reduction of targeted bands, producing an artifactual result that may be interpreted as a polymorphism.

The apparent lack of stringency in the primer-template interaction, however, can be capitalized upon for the design of entirely new applications of PCR technology. In addition to isolating unknown stretches of contiguous genomic DNA, the procedure can be used to isolate genes from cDNA, plasmid or phage libraries, or from any starting single or double stranded DNA template for which a limited amount of sequence information is known. The method we have described can be adapted to search for polymorphisms adjacent to known genes (e.g. tumor suppressor genes, oncogenes) by combining the 'gene-walking' procedure with an internal oligomer extension protocol that uses radiolabeled nucleotides during the extension step rather than a kinased 'internal' primer. The labeled extension product can be digested with a series of restriction enzymes to search for polymorphisms in the target gene.

The examples presented in this report and applications described above are the results of a single step 'gene-walking' PCR protocol, and as such, the average product size would be

generally limited to under 5 kilobases. Some of the more important uses of this procedure, however, will involve the use of serial 'gene-walking' to amplify and characterize much longer genomic sequences. For example, the largest band that is identified by oligomer-extension to be a targeted PCR product of gene-walking can be excised, sequenced using the 'walking' primer until 30–40 nucleotides of unique sequence are identified, and a second 'targeted' and 'internal' detection primer could be constructed from this sequence. A repeat of the 'gene-walking' PCR method would result in 2–5 kb serial 'walks' along the chosen direction of the gene in question, without the need for intervening cloning or screening steps. The repetitive application of this procedure can further be adapted for automated sequence analysis.

Although the method as described utilizes agarose gel electrophoresis for the identification and isolation of the amplified sequence of interest, the method can be readily modified for use with affinity based hybrid capture technology (e.g. by using a biotinylated internal primer attached to an avidin coated matrix) to extract the 'walking' product after PCR (17). This would eliminate the need for the electrophoresis step, further reducing the time required for isolating the amplified sequence, and making the procedure more amenable to automation.

For the applications described above, the apparent lack of a requirement for an absolutely specific primer-template interaction serves as an advantage by generating a large number of variably sized products for analysis. A number of potential applications exist for 'gene-walking' PCR, however, in which the detection of specific sequences may be desirable, for example, when searching for regulatory regions or restriction sites for mapping purposes. Our data suggests that strategies such as increasing annealing temperature will be necessary in order to improve the specificity of hybridization. The length of the product can then be further modified by increasing the duration of extension during the PCR reaction.

The 'gene-walking' method, combining PCR with oligomer-extension, will provide a powerful new approach for the isolation of genes that have hitherto required laborious cloning, screening, or Southern hybridization methods. Serial application of 'gene-walking' PCR should assist in the rapid analysis of linear sequences of unknown regions of genomic DNA that are adjacent to known genes.

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