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Endonuclease-Mediated Long PCR and Its Application to Restriction Mapping

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The polymerase chain reaction (PCR) is the most widely used technique for the study of DNA. Applications for PCR have been extended significantly by the development of "long" PCR, a technique that makes it possible to amplify DNA fragments up to 40 kb in length. This article describes two novel applications of the long PCR technique, one which simplifies restriction mapping and another which enhances amplification specificity and yield. The same primers used to perform the long PCR amplification can be used as probes to perform restriction mapping of the DNA fragment amplified. Restriction digestion performed prior to long PCR amplification can be used to selectively suppress the amplification of members of families of closely related DNA sequences, thereby making it possible to selectively amplify one of a group of highly homologous sequences. These two complimentary techniques, both involving use of the long PCR paired with restriction digestion, have potential application in any laboratory in which PCR is performed.

Introduction

The polymerase chain reaction (PCR) has become the most widely used technique for the study of DNA; with applications pertaining to cDNA cloning, gene cloning, polymorphism detection, mutagenesis and allele-specific diagnosis among many others (1, 20). Each of these applications has been extended by the development of "long" PCR (2, 3), a technique which makes it possible to amplify DNA fragments up to 40 kb in length. This article will describe two applications in which long PCR has been paired with DNA restriction endonuclease digestion. In one case, the approach serves to simplify restriction mapping, a technique central to DNA characterization. The second approach involves performing restriction digestion prior to PCR amplification to "selectively suppress" the amplification of highly homologous DNA sequences.

Restriction mapping is a very useful step in the characterization of a large DNA molecule. DNA mapping by restriction enzyme digestion has been used routinely in preparation for subcloning, in the analysis of restriction fragment length polymorphisms, for comparing different genomic DNA clones, and in the construction of physical maps of chromosomes (4). Several different approaches have been used to perform restriction mapping (5-8). The "classical" approach involved digestion of the DNA fragment, initially with a single restriction enzyme and, subsequently, with a combination of enzymes, followed by gel electrophoresis (5).

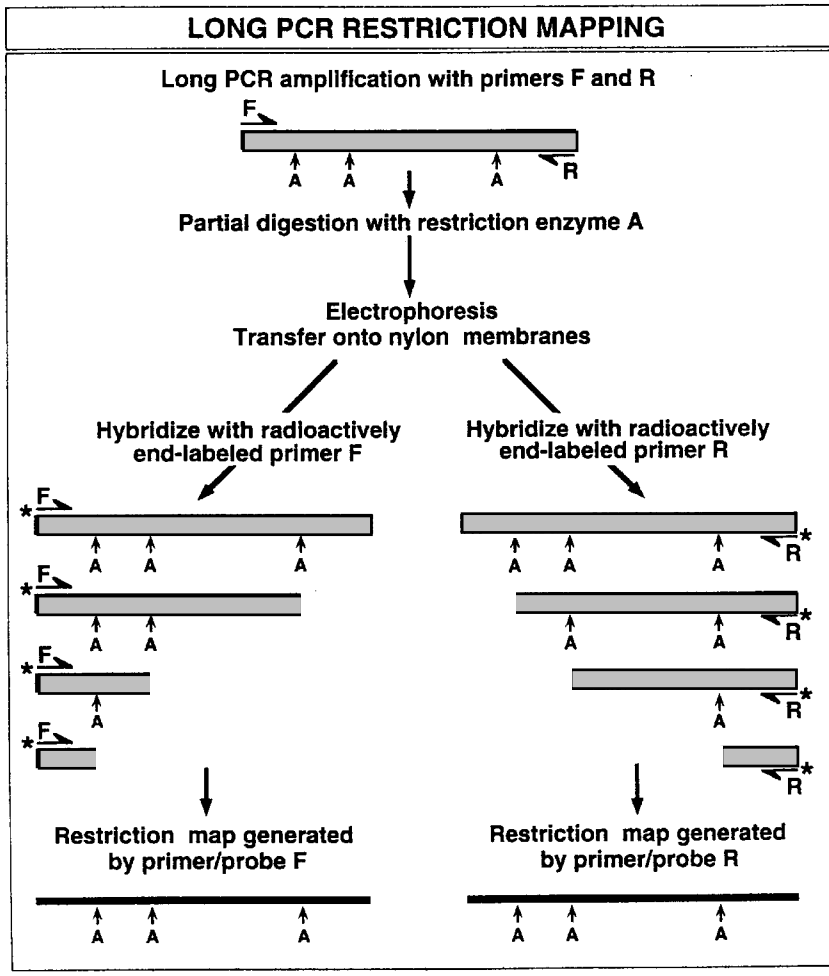

Figure 1. Long PCR-based rapid DNA restriction mapping. Please see text for details.

A significant improvement was made by Smith *et al*. when they suggested that the DNA fragment of interest be radioactively labeled at both ends with ^{32}P using polynucleotide kinase and ^{32}P-γ-ATP (6). The end-labeled DNA was then digested with an enzyme for which there was only a single restriction site on the target sequence, and the two resulting fragments were separated by gel electrophoresis. These two DNA fragments, each labeled at one end, were then subjected to partial restriction digestion with different restriction enzymes. The resultant overlapping restriction fragments, all with a common radioactively labeled terminus, were separated by gel electrophoresis and detected by autoradiography.

Another restriction mapping technique involved partial digestion of genomic DNA that had been subcloned into lambda phage or a cosmid vector, followed by Southern analysis performed with radioactively labeled oligonucleotide probes which had been designed on the basis of known vector sequence. Generation of this type

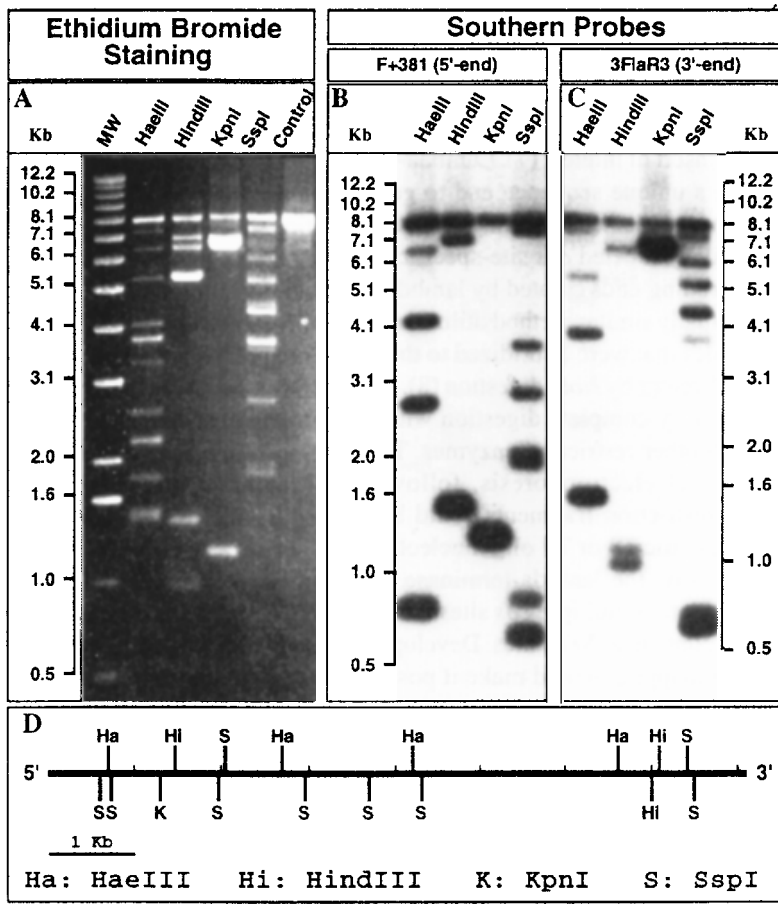

Figure 2. Long PCR-based rapid DNA restriction mapping of a portion of the human estrogen sulfotransferase gene (9, 10). Panel A shows ethidium bromide stained DNA fragments obtained after partial restriction digestion of a long PCR amplification product, approximately 8.1 kb in length, obtained with human genomic DNA as template. Partial restriction digestion was performed by adding 2 units of either *Kpn*I or *Hae*III, 20 units of *Hind*III, or 6 units of *Ssp*I individually in 2 µl aliquots to separate microcentrifuge tubes that contained 40 µl of the PCR amplification mixture. Restriction reactions were performed as described in the text. Panels B and C show Southern blot analysis performed with radioactively end-labeled forward and reverse long PCR primers as probes after transferring partially digested restriction fragments to nylon membranes. Some of the bands seen in panel A are not radioactively labeled because they do not include the primer sequences. Panel D shows the restriction map of the human estrogen sulfotransferase amplification product that was deduced from the Southern blots. "MW" represents a Gibco BRL 1 kb DNA ladder, and "Control" represents undigested amplification product. Reproduced with permission of Eaton Press (9).

of restriction map often began with lambda-terminase digestion of a cosmid that contained the insert of interest (7). Lambda-terminase cleavage was used to linearize the cosmid at a unique sequence and to generate protruding ends. The linearized cosmid was then subjected to partial restriction digestion, followed by hybridization with radioactively labeled *cos* site-specific oligonucleotides that hybridized to the 12 bp 5'-protruding ends created by lambda-terminase digestion (7).

A conceptually similar method utilized radioactively labeled T3- and T7-specific oligonucleotides that were hybridized to the ends of an insert that had been excised from a cosmid vector by *Not*I digestion (8). After the DNA insert had been separated from the vector by complete digestion with *Not*I, it was then subjected to partial digestion with other restriction enzymes. The resulting restriction fragments were separated by gel electrophoresis, followed by transfer to a membrane. The immobilized restriction fragments could be detected by the use of radioactively labeled end-specific T3 or T7 oligonucleotides (8).

Unfortunately, the lambda-terminase technique is not suitable for mapping cosmids that contain multiple *cos* sites, and the T3-T7-specific technique requires that the insert contain no *Not*I sites. Development of a vector-independent procedure for restriction mapping would make it possible to obtain restriction maps without the requirement that vector sequence be known, and, of more importance, without the requirement for subcloning. Development of the long PCR now makes such an approach feasible (2, 3).

Long PCR-based restriction mapping can potentially be used to generate restriction maps quickly and simply for any DNA sequence that can be amplified (9), which, for the long PCR, includes regions up to 40 kb in length (2, 3). The technique utilizes the same general principles used in the 3'- and 5'-labeling of restriction fragments generated from either purified DNA molecules or sequences which have been cloned into a vector. However, the long PCR approach does not require purification or cloning of the DNA molecule to be characterized, and it does not require knowledge of a vector sequence. The technique is illustrated schematically in Figure 1. The same primers used to perform the PCR amplification are radioactively labeled so they can also serve as probes for the detection of DNA fragments generated by partial restriction enzyme digestion of the long PCR amplification product. The method has been used successfully to generate restriction maps for the human estrogen sulfotransferase gene (9, 10) and the human thiopurine methyltransferase gene (11). Application of this long PCR-based technique for restriction mapping of the human estrogen sulfotransferase gene is shown in Figure 2 (9, 10). A different, but related application of long PCR also pairs the technique with restriction digestion, but in this case restriction digestion is performed before the amplification reaction to make possible the selective amplification of a single member of a family of highly homologous DNA sequences (12).

High specificity and adequate yield of the desired amplification product are important issues in all PCR reactions, including long PCR amplifications. Many different strategies have been used in attempts to increase reaction yield and specificity. For example, considerable effort has been expended on the optimal design of PCR primers and on the modification of reaction conditions, (1, 13, 14). "Selective suppression" by restriction endonuclease digestion is a long PCR technique that is intended to enhance both specificity and yield when the long PCR is used to amplify individual members of families of closely related DNA sequences. We tested the feasibility of this approach by using it to amplify individual members of a group of three highly homologous human chromosome 16 phenol sulfotransferase genes, *STP1*, *STP2* and *STM* (12, 15, 16). Selective suppression combines pre-amplification restriction endonuclease digestion with a hot start for the long PCR, all performed in a single reaction tube. Reagents used for restriction digestion of template DNA are initially separated by a wax barrier from those required for the long PCR amplification. After the hot start of the amplification reaction, the final concentrations of all the reactants are optimal for performance of long PCR. However, DNA template sequences which might have resulted in generation of nonspecific amplification products have been eliminated by restriction digestion performed in the same reaction tube immediately prior to the long PCR reaction. Furthermore, since template sequences which might compete for primers and other limiting reagents during the long PCR have been removed prior to the reaction, the selective suppression technique

also promotes increased yields of the desired amplification product. This approach is especially useful when it is not possible to design optimal primers, either as a result of limited knowledge of the DNA sequence to be amplified or in situations in which multiple copies of very similar sequences are present in the template DNA.

Restriction digestion has been used previously as a way to remove contaminating DNA prior to the addition of template (17) or as a method to enhance the amplification of relatively short, known sequences (18). However, in selective suppression of long PCR, the amplification of entire genes or large portions of genes is often the goal, and much of the amplified sequence may be unknown. The most appropriate endonuclease for a given application can be selected on the basis of either the results of pilot experiments or knowledge of restriction patterns for one or more of the highly related sequences to be amplified. This strategy reduces the effort required to design specific PCR primers for the amplification of very similar sequences, and it is particularly useful when limited sequence information is available for primer design.

We have illustrated the selective suppression technique in Figure 3 by showing its use for the specific amplification of two of the three phenol sulfotransferase genes found on human chromosome 16 (15, 16). The figure shows both a schematic outline of the technique (Figure 3A) and actual data for an amplification performed after selective suppression by *Eco*RI or *Xba*I digestion (Figure 3C). Because of the very high degree of sequence identity present among these genes, a single primer pair can be used to amplify *STP1*, *STP2* and *STM*. The particular pair of primers used to obtain the data shown in Figure 3 amplifies the coding exons of all three genes in fragments 7.5, 7.0 and 6.9 kb in length for *STP2*, *STM* and *STP1*, respectively (12, 15, 16; Figure 3). However, *STP2* is not cleaved by *Xba*I restriction digstion, while both *STP1* and *STM* are cut by this enzyme (see the table in Figure 3B). Conversely, *STM* has no restriction sites for *Eco*RI, while *STP1* and *STP2* both contain *Eco*RI restriction sites. The enhanced specificity and yield of *STM* amplification after prior restriction digestion with *Eco*RI, and the enhanced specificity and yield of the *STP2* amplification product following incubation with *Xba*I are shown in Figure 3C. In essence, this approach converts the high degree of sequence similarity present within members of some gene families from a disadvantage into an advantage. Use of selective suppression makes it possible to study individual members of gene families with highly similar sequences by "spotlighting", in turn, each member of the family (Figure 3). Therefore, selective suppression, a technique that involves restriction endonuclease digestion performed in the same reaction tube prior to long PCR amplification, like the use of the long PCR to simplify and speed restriction mapping, should have potential application in any laboratory in which the PCR is performed.

Protocols

Required Reagents and Equipment

AmpliWax PCR Gem 100 beads (Perkin-Elmer, Norwalk, CT); DNA markers (Gibco BRL, Life Technologies, Gaithersburg, MD); ExpressHyb hybridization solution (Clontech Laboratories, Inc., Palo Alto, CA); Quick Spin Columns, Sephadex G-25, Fine (Boehringer Mannheim, Indianapolis, IN); GeneAmp XL PCR Kit (Perkin-Elmer); MSI nylon membranes (Micron Separations Inc., Westboro, MA); restriction enzymes (New England Biolabs, Beverly, MA); Southern blot transfer chamber; submarine type electrophoresis apparatus; T4 polynucleotide kinase (Gibco BRL, Life Technologies); thermal cycler (Cetus Model 480, Perkin-Elmer); 0.5 ml thin-walled reaction tubes (Perkin-Elmer).

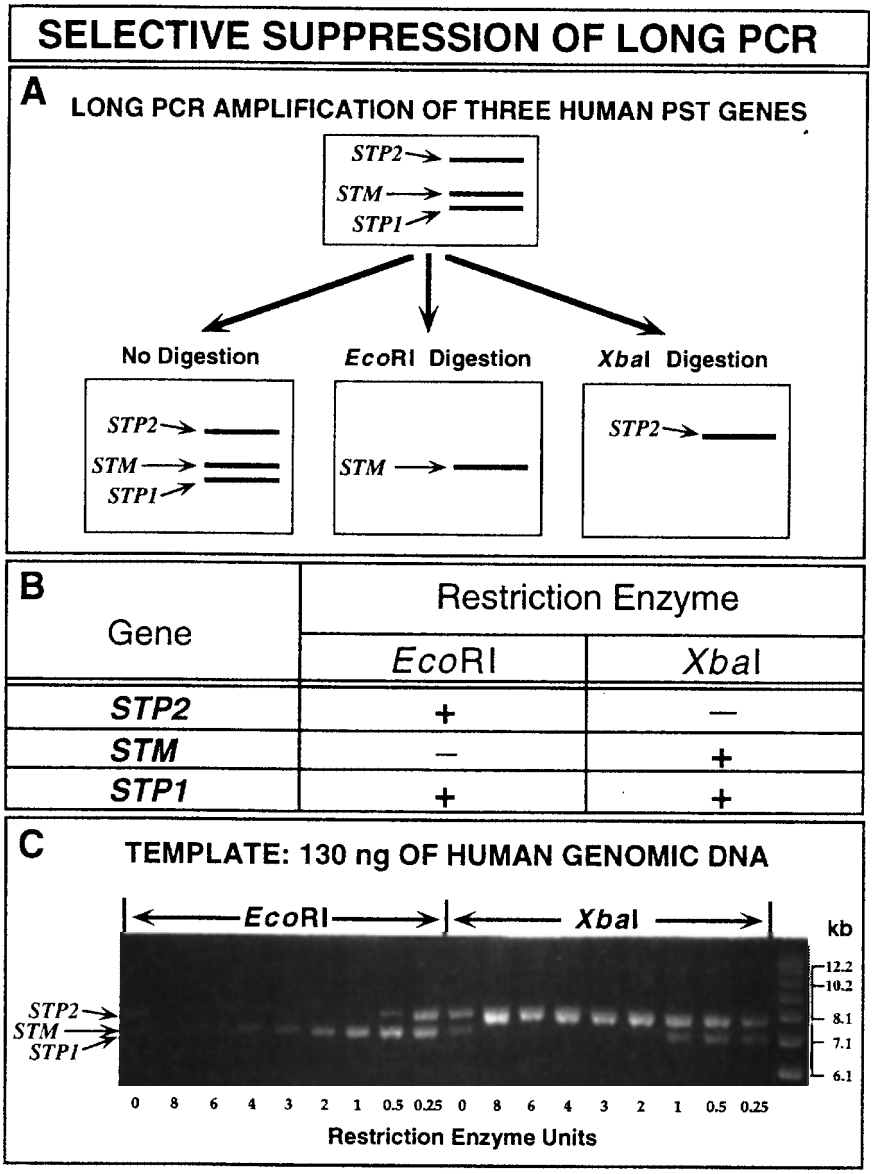

Figure 3. Selective suppression of the long PCR by restriction endonuclease digestion. (A) A schematic diagram of selective suppression of the long PCR as applied to the human phenol sulfotransferase genes *STP1*, *STP2* and *STM* is shown. These three human chromosome 16 genes can each be amplified by use of the primers F2 and R2 (12, 15, 16). The effect of digestion of human genomic DNA with either *Eco*RI or *Xba*I prior to long PCR amplification of these genes is depicted schematically. (B) Susceptibility of human phenol sulfotransferase genes *STP2*, *STM* and *STP1* to restriction digestion with *Eco*RI and *Xba*I. A (+) in the table indicates that the portion of the gene amplified by long PCR primers F2 and R2 can be digested by the enzyme, while a (-) indicates that it cannot be digested. (C) Effect on the amplification of *STP2*, *STM*, and *STP1* of selective suppression performed with varying concentrations of *Eco*RI and *Xba*I, using human genomic DNA as the template. *STM* and *STP1* cannot always be resolved clearly.

Protocol 1: Long PCR-Based Restriction Mapping

Long PCR reaction
All reagents for long PCR except primers and template are supplied with the GeneAmp XL PCR Kit or similar kits that can be obtained from other suppliers. Please refer to the GeneAmp XL PCR Kit instructions for additional instructions.

1. Prepare master mixtures of the lower and upper PCR buffers in two sterile microcentrifuge tubes. Quantities of reagents for a single reaction are listed below. Multiply by the desired number of reactions. Mix well by vortexing.

Lower buffer		*Upper buffer*	
1.6 µl	Forward primer (10 pmol/µl)	18 µl	3.3x XL buffer
1.6 µl	Reverse primer (10 pmol/µl)	1 µl	DNA template (80-160 ng/µl)
12.0 µl	3.3x XL buffer	2 µl	r*Tth* DNA polymerase, XL
4.4 µl	25 mM $Mg(CH_3CO_2)_2$	39 µl	ddH_2O
8.0 µl	dNTPs (2.5 mM each)		
12.4 µl	ddH_2O		

2. Program the thermal cycler as follows:

File 1	80˚C for 5 min;	25˚C for at least 2 min;	1 cycle
File 2	94˚C for 1 min;		1 cycle
File 3	94˚C for 30 s,	66˚C for 10 min;	16 cycles
File 4	94˚C for 30 s,	66˚C for 10 min, with 15 s incremental lengthening step for each cycle	12 cycles
File 5	72˚C for 10 min;		1 cycle
File 6	4˚C hold		

   The temperature during the denature/annealing step must be adjusted on the basis of the properties of the primers used.
3. Pipette 40 µl of the lower PCR buffer into the bottom of a 0.5 ml thin-walled PCR tube.
4. Add one AmpliWax PCR Gem 100 bead to the PCR tube.
5. Place the PCR tubes in the thermal cycler. Heat at 80˚ C for 5 min and then cool to 25˚C (i.e. initiate thermal cycler file 1, as outlined in step 2).
6. Pipette 60 µl of the upper PCR buffer into the PCR tubes at 25˚C.
7. Proceed with the remainder of the PCR cycle as outlined in step 2.

Partial Restriction Enzyme Digestion
1. Decide how many restriction enzymes are to be used for restriction mapping. Prepare two sets of microcentrifuge tubes for each enzyme and pipette 40 µl of the PCR reaction mixture into one set of microcentrifuge tubes on ice.
2. Pipette 3 µl of 0.5 M EDTA, pH 8.0, into the other set of microcentrifuge tubes. These tubes will be used to terminate the restriction digestion reaction.
3. Add 2 µl of the restriction enzyme to be tested to the microcentrifuge tubes with the PCR mixture. Mix well, centrifuge briefly and then incubate at the appropriate temperature. (See Notes and Tips 2).
4. Terminate the restriction reactions after 2.5, 7.5, 15 and 30 min incubations by transferring 10 µl of the reaction mixture for each restriction enzyme into the tube containing EDTA for that enzyme (described in step 2) In other words, all four timepoints for each enzyme will be pooled in a single tube, increasing the probability that *all* possible products of the partial digestion will be represented.
5. Mix well by vortexing and then centrifuge briefly.

Electrophoresis

1. Add an appropriate quantity of gel-loading buffer to the partially digested DNA sample. Place 20 μl of each sample in a 0.8% agarose gel cast in 1x TAE. A duplicate series of samples should be analyzed on the other side of the gel to be probed with the alternative primer.
2. Perform electrophoresis overnight in 1x TAE buffer at 1 volt/cm.

Southern Blot

1. Stain the gel with ethidium bromide and photograph under UV light with a fluorescent ruler placed beside the DNA marker lane.
2. Prepare the gel for capillary transfer after trimming as follows:
   (i) Soak the gel in 500 ml 0.25 M HCl for 15 min and then rinse with ddH_2O.
   (ii) Soak the gel in 500 ml 0.5 M NaOH in 1.5 M NaCl for 15 min, and then rinse with ddH_2O.
   (iii) Repeat step (ii) two times.
   (iv) Soak the gel in 500 ml 0.5 M Tris-HCl, pH 7.5 in 1.5 M NaCl for 15 min, and then rinse with ddH_2O.
   (v) Repeat step (iv).
3. Cut an MSI nylon membrane to a suitable size, and soak the membrane for 15 min in 5x SSPE .
4. Transfer DNA from the gel to the nylon membrane overnight by capillary action in 20x SSPE.

Radioactive Labelling of Primers

1. Prepare reagents for T4 polynucleotide kinase reactions for the two long PCR primers in two microcentrifuge tubes as listed, and then incubate at 37˚C for 30 min.

3 μl	oligonucleotide primer (10 pmol/μl)
2 μl	10x kinase reaction buffer
2 μl	^{32}P-γ-ATP (7000 Ci/mmol)
2 μl	T4 polynucleotide kinase
11 μl	ddH_2O

2. Inactivate the T4 polynucleotide kinase by incubation at 97˚C for 2 min.
3. Purify the labeled oligonucleotides with Sephadex G-25 Quick Spin Columns according to the manufacturers directions.
4. Use 1 μl of the purified primer to determine the specific activity.

Hybridization

1. Rinse the nylon membrane from "*Southern Blot*" step 4 in 5x SSPE. Air dry the membrane on Whatman paper, and then vacuum-dry the membrane at 80˚C for 1.5 hr.
2. Prehybridize the membrane at 37˚C for 1 hr in ExpressHyb hybridization solution (approx. 0.2 ml/cm^2).
3. Discard the original ExpressHyb solution and add an identical volume of fresh ExpressHyb solution and a suitable quantity of the probe (1-2 x 10^6 cpm/ml). Mix well and incubate at 37˚C for 1 hr.
4. Wash the membrane three or more times at room temperature in 2x SSC with 0.05% SDS. If there is more than one membrane, each membrane should be washed separately.
5. Wrap each membrane in a plastic bag and expose it to an x-ray film to obtain an autoradiogram.

Protocol 2: Selective Suppression of Long PCR

Restriction Digestion and Long PCR Reactions

1. Prepare a "master mixture" of the lower PCR buffer in a sterile microcentrifuge tube. Quantities of reagents required to perform a single reaction are listed below. Multiply by the desired number of reactions and mix well by vortexing.

Lower buffer	
1.6 µl	Forward primer (10 pmol/µl)
1.6 µl	Reverse primer (10 pmol/µl)
23.4 µl	3.3x PCR buffer
8.0 µl	dNTPs (2.5 mM each)
43.4 µl	ddH_2O

2. Program the thermal cycler as follows:

File 1	80˚C, 5 min	25˚C, at least 2 min	37˚C, 20 min	1 cycle
File 2	94˚C, 1 min			1 cycle
File 3	94˚C, 30 s	66˚C, 10 min		16 cycles
File 4	94˚C, 30 s	66˚C, 10 min with 15 s incremental lengthening step for each cycle		12 cycles
File 5	72˚C for 10 min			1 cycle
File 6	4˚C hold			

3. Pipette 78 µl of the lower PCR buffer into a 0.5 ml thin-walled PCR tube.
4. Add one AmpliWax PCR Gem 100 bead to each PCR tube.
5. Place the reaction tubes in the thermal cycler.
6. Prepare the upper PCR buffer as listed below:

Upper buffer	
6.6 µl	3.3x XL buffer
4.4 µl	25 mM $Mg(CH_3CO_2)_2$
2.0 µl	r*Tth* DNA polymerase, XL
2.0 µl	Restriction enzyme at an appropriate concentration
1.0 µl	DNA template (80 - 160 ng/µl)
6.0 µl	ddH2O

   Mix well by pipeting up and down. Restriction enzymes are diluted in 1x restriction enzyme buffer and should be added to the upper buffer mixture immediately before the PCR cycle is initiated.
7. Initiate the PCR cycle outlined in step 2.
8. During the thermal cycler File 1 25˚C incubation, add 22 µl of the upper PCR buffer over the wax barrier.

Electrophoresis

1. Analyze 10 µl of each amplification product by agarose gel electrophoresis.

Notes and Tips

1. Primer sequences for long PCR reactions should be examined carefully for secondary structures and false priming by the use of primer design computer software, such as NBI's Oligo (National Biosciences, Inc., Plymouth, MN) (13). Pilot experiments may be needed to determine optimal PCR conditions, since those conditions will be primer-dependent.

2. It is recommended that a pilot experiment be performed to determine the appropriate amount of a particular restriction enzyme that is required to obtain partial digestion. Many restriction enzymes possess variable activities when restriction digestion is performed in the PCR reaction mixture (19). We have found it convenient to perform a series of restriction digestion reactions with different concentrations of a particular restriction enzyme while holding restriction digestion time constant.
3. Eighty to 160 ng of human genomic DNA should be adequate for the long PCR amplification of a 10 kb target sequence.
4. Restriction maps generated from either end of a target DNA fragment with a particular restriction enzyme should agree (Figure 1), especially within the central portion of the target sequence. Lack of consistency between these results usually reflects nonspecific hybridization of the probes to the partially digested DNA fragments. This problem can be addressed by: (a) increasing hybridization temperature; (b) using a highly stringent wash after the hybridization; or (c) using a probe that is located internal to the PCR amplification primer, to achieve greater specificity.

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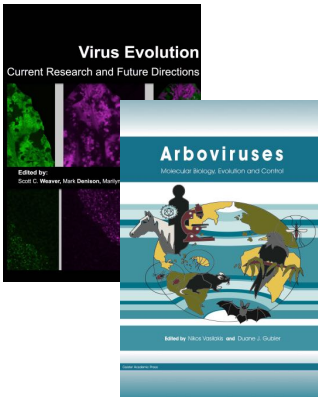

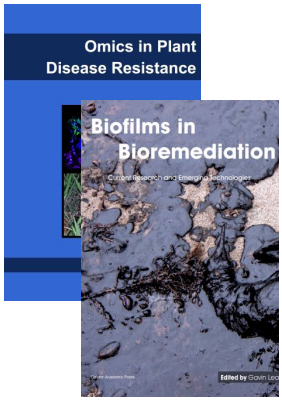

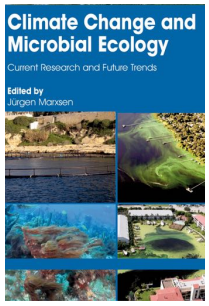

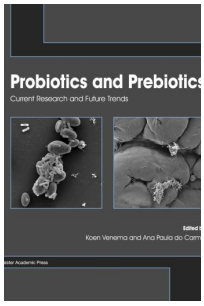

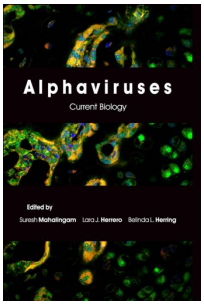

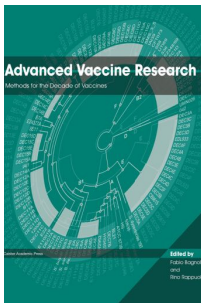

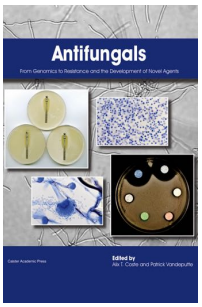

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