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A PCR-based Method for Isolation of Genomic DNA Flanking a Known DNA Sequence

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We describe a simple PCR-based method for the isolation of genomic DNA that lies adjacent to a known DNA sequence. The method is based on the directional cloning of digested genomic DNA into the multiple cloning site of a pUC-based plasmid to generate a limited genomic library. The library is plated onto a number of selective LA plates which are incubated overnight, and recombinant plasmid DNA is then isolated from resistant colonies pooled from each plate. PCR amplification is performed on the pooled recombinant plasmid DNAs using primers specific for the pUC vector and the known genomic sequence. The combination of efficient directional cloning and bacterial transformation gives relative enrichment for the genomic sequence of interest and generates a simple DNA template, enabling easy amplification by PCR.

Introduction

The polymerase chain reaction (PCR) is a powerful technique for highly specific amplification of DNA defined by two flanking primers and has had a major impact on many aspects of biology (1). In the past, one of the factors limiting its application has been the requirement that both sequences flanking the DNA of interest must be known to specify the primers to be used. A number of methods have since been reported that overcome this limitation and allow PCR to be used to amplify DNA outside a region of known sequence. The first protocol described, termed Inverse PCR, involves digestion of the DNA to produce a fragment containing known sequence, followed by circularization by intramolecular ligation (2). The resulting circular template is then PCR amplified via the use of divergent primers which have been designed to anneal within the region of known sequence. This method is only suitable when the digested DNA can be monomerically circularised. In Capture PCR, only one primer is derived from the known sequence, whereas the second primer anneals to a linker which has been ligated to one end of the digested DNA sample. The second primer is also biotinylated to enable isolation of subsequent extension products on a streptavidin support (3). Ligation of a linker to allow the use of a defined second primer is also the strategy employed in Ligation Mediated PCR, which is a method that has been used to generate *in vivo* footprints of transcription factors bound to DNA (4). In this case, the linkers are ligated to blunt ends which have been generated by random chemical cleavage of the genomic DNA template, followed by denaturation of and primer extension from a known sequence. Two further methods rely on the use of less specific second primers: Restriction-

Site PCR employs oligonucleotide primers specific for given restriction enzyme recognition sequences, in conjunction with a specific primer derived from the known DNA sequence (5), whereas Targeted Gene Walking is based on PCR initiation with primers bearing only partial homology at the 3' end of the target sequence (6). The disadvantage of this latter method is that it produces numerous non-specifically amplified species in addition to the correct PCR product.

The protocol we present here is most similar to the method described by Mizobuchi and Frohman 1993, called Rapid Amplification of Genomic DNA Ends (RAGE) (7). Both approaches involve ligation of digested genomic DNA to plasmid DNA. PCR primers are designed so that one is homologous to a known target region in the genomic DNA sequence, and the other is homologous to a defined plasmid sequence. By the RAGE method, cloning is accomplished via digestion of genomic DNA with a single restriction endonuclease, and the products are ligated into the linearized, phosphatased plasmid vector. PCR is performed directly on the ligated DNA employing nested primers and two rounds of amplification to increase specificity. In contrast, our method involves directional cloning of genomic DNA into the multiple cloning site of a plasmid vector. The ligated products are then transformed into bacteria and plated on selective media, and the plasmid DNA recovered from the pool of surviving clones is PCR amplified. These additional straight-forward steps give relative enrichment for the genomic sequence of interest and generate a simple DNA template, enabling easy amplification by PCR.

Protocol

Required Reagents and Suggested Suppliers

Enzymes and Plasmids
Restriction enzymes (Boehringer Mannheim), T4 DNA Ligase (Boehringer Mannheim), *Taq* Polymerase (Bioline). pUC18 (Boehringer Mannheim), pSL1180 (Pharmacia).

Other Reagents
Electroporation cuvettes, 2 mm gap (Biorad), Oligonucleotides: Universal primer (pUC18) 5'ACACAGGAAACAGCTATGACCATG 3', TA Cloning Kit (Invitrogen).

Protocol
The basic steps in the protocol are outlined in Figure 1.

Digestion of Genomic and Plasmid DNA with Suitable Restriction Enzymes
1. Digest to completion 10 μg genomic DNA in a volume of 200 μl with 30 units of the appropriate restriction enzymes, according to the manufacturer's instructions.
2. Digest to completion 1 μg plasmid DNA in a volume of 30 μl with suitable restriction enzymes (i.e. enzymes which will allow directional cloning of the inserts generated in step 1). Isolate the vector fragment away from the excised polylinker by gel electroelution, phenol:chloroform extraction and ethanol precipitation and resuspend in 20 μl sterile water.

Ligation of Genomic and Plasmid DNA
1. Set up the ligation with 300 ng genomic DNA and 50 ng plasmid vector in a volume of 10 μl using 0.8 units T4 DNA ligase (Boehringer Mannheim) and manufacturer's ligation buffer. Incubate at 16°C overnight.

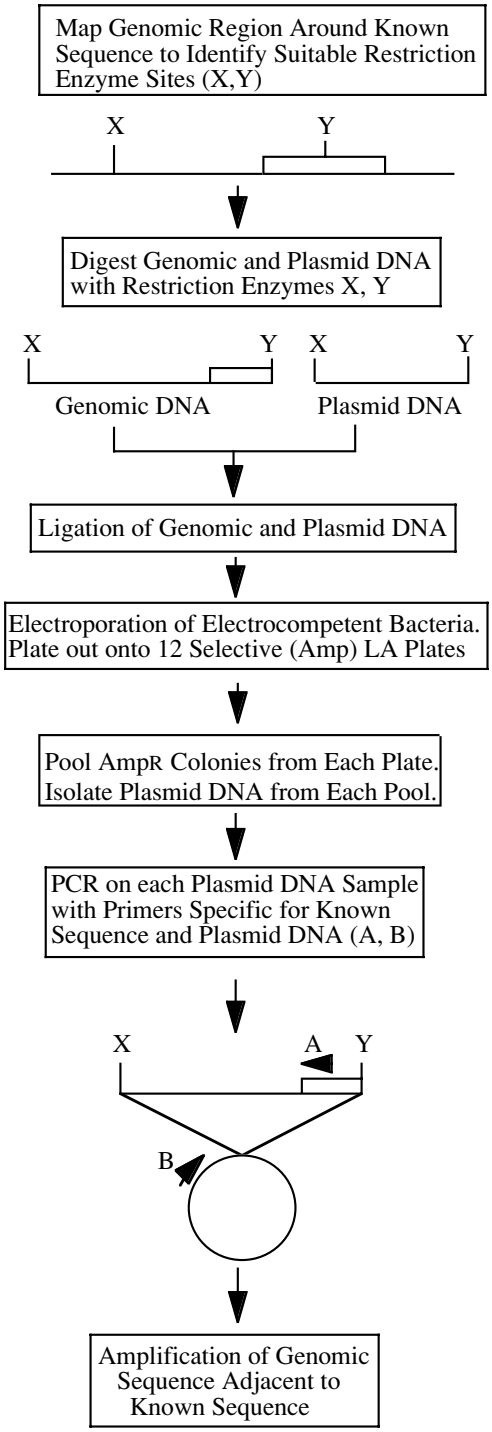

Figure 1. A flow chart outlining the basic steps of the PCR-based protocol to isolate genomic DNA flanking a known DNA sequence.

Electroporation and Plating of Transformed Bacteria

1. Ethanol precipitate the ligated DNA and resuspend in 5 μl sterile water.
2. Electroporate 2.5 μl of the ligation mix per 40 μl of electrocompetent *Escherichia coli*, prepared according to Dower (8), using a Biorad Gene Pulser set at 200 μF, 25 and 2.4 kV and electroporation cuvettes with a gap of 2 mm.
3. Add 1 ml SOC medium and allow bacteria to recover by shaking for 1 hour at 37°C.
4. Plate onto twelve 150 mm diameter LA plates containing 100 μg/ml ampicillin and incubate overnight at 37°C.

Isolation of Plasmid DNA and Setting Up the PCR

1. Pool all of the ampicillin resistant (AmpR) colonies on each of the twelve plates, add 10 mls SOC medium with 100 μg/ml ampicillin and shake for 4 hours at 37°C.
2. Spin down the cells at 4,000 rpm for 10 minutes and isolate the plasmid DNA according to Sambrook *et al*. (9).
3. Set up the PCR reactions with 10 ng plasmid DNA and 25 pmol of each primer: one specific for the known genomic sequence, and the other specific for the vector (for example the universal primer if using pUC-based plasmids 5' ACACAGGAAACAGCTATGACCATG 3'). Reaction volume is 50 μl, containing 2 units of *Taq* polymerase and the buffer supplied by the manufacturer (5 mM KCl, 1 mM Tris pH 7, 0.01 mg/ml gelatin, 3 mM $MgCl_2$). The PCR conditions used will depend on the specific set of primers and the size of fragment to be amplified. In the experiment for which the protocol was developed (10) these were: 95°C for 5 min followed by 93°C for 30 s, 65°C for 50 s, 70°C for 6 min, repeated three times; 93°C for 30 s, 64°C for 50 s, 70°C for 6 min, 93°C for 30 s, 63°C for 50 s, 70°C for 6 min, 93°C for 30 s, 62°C for 50 s, 70°C for 6 min, 93°C for 30 s, 61°C for 50 s, 70°C for 6 min, 93°C for 30 s, 60°C for 50 s, 70°C for 6 min, repeated 30 times.

Isolation and Cloning of the PCR Product

1. Run the PCR reactions on a 0.8% agarose gel.
2. Isolate the PCR product of the expected size by electroelution and clone and/or nucleotide sequence to characterize further. The PCR product can be cloned using the TA Cloning Kit (Invitrogen); if a single product is obtained in the PCR, it can be cloned directly, without the need for electroelution.

Notes and Tips

Digestion of Genomic and Plasmid DNA with Suitable Restriction Enzymes

There is no need to size select the genomic DNA after digestion, prior to ligation. The use of two different restriction enzymes allows highly efficient directional cloning, gives enrichment for the correct insert and precludes the need to phosphatase the vector. The choice of plasmid will be influenced by the suitable restriction enzyme sites identified by mapping the genomic locus. There are many pUC-based plasmids available, including pSL1180 (Pharmacia) which has more than thirty different restriction enzyme sites in its multiple cloning site. It is worthwhile to purify the digested vector away from the excised part of the multiple cloning site, as this will reduce background due to vector religation.

Electroporation and Plating of Transformed Bacteria

Prior to electroporation, it may be advantageous to digest the ligated DNA with an enzyme whose site is absent from the target genomic insert and plasmid vector. This will enrich the ligation mixture for plasmids containing the target insert by linearizing any plasmids containing the restriction endonuclease site, as bacterial transformation of linear DNA is extremely inefficient.

Concluding Remarks

The method we describe provides a rapid and easy means to isolate genomic sequences that lie adjacent to known DNA sequences. The strength of this protocol lies in its simplicity, and in the selection steps which enrich for a specific region of interest in a highly complex genome. Only a preliminary Southern blotting analysis needs to be performed to obtain a simple map of the region flanking the piece of known DNA. By using directional cloning, which is very efficient, there is no need to size fractionate the genomic DNA, and enrichment for a particular genomic sequence can be achieved by the use of suitable restriction enzymes. This enrichment step is generally applicable, since the selection of appropirate restriction enzyme sites is only limited by their presence in the multiple cloning site of the plasmid vector; given that there are many plasmid vectors which have large multiple cloning sites [for example, pSL1180 (Pharmacia)] this is generally not problematic. Finally, the primers and template used in the PCR reaction can be selected so that specific restriction enzyme sites are present at each end of the molecule, and these sites can be chosen to be optimal for efficient cloning and PCR.

The ability to amplify DNA segments that lie outside the boundaries of known sequence has a number of applications in molecular biology. These include walking along genomic DNA to sequence regions upstream of cloned cDNAs, isolating unknown stretches of contiguous genomic DNA, and identifying restriction sites for mapping purposes and DNA footprint analysis. A further application is in cloning genes by insertional mutagenesis, an approach that relies on the integration of foreign DNA into the genome causing disruption of an endogenous gene, resulting in a mutant phenotype (11). The disrupted gene can then be cloned by using the introduced DNA as a molecular tag. This is a particularly powerful approach when combined with transgenic mouse technology, since the consequences of the resulting mutation can be studied within the context of the whole animal. The method we describe here was originally developed for this application, and has been successfully used to isolate genomic sequences flanking a retroviral vector integrated into the mouse genome (10). The method has a number of advantages for cloning genomic DNA disrupted by insertional mutagenesis. By choosing appropriate restriction enzymes, it is possible to clone one particular insertion site if several exist for a given piece of foreign DNA. Enrichment for a site of insertion can also be achieved if a rare restriction enzyme site, such as *Not* I, is present in the foreign DNA used for the mutagenesis experiment. As the same foreign DNA can be used in different insertional mutagenesis experiments, the same primers and PCR conditions can be employed to isolate different insertion sites; only the restriction enzymes used for digestion of the genomic DNA need to be determined for each site.

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