arise in using the two-hybrid system. BioTechnology 14:920-924.

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Generation of Vector-Insensitive Dig-Labeled Probes from Large Cosmid Library Inserts Using PCR

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ABSTRACT

We have devised a general method for producing vector-insensitive probes from clones in which insert DNA (ca. 40 kb) could not be amplified in one piece nor be excised from the vector sequence. The method involves PCR and vector-specific primers in combination with restriction digestion and ligation. It yields specific PCR

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products that could subsequently be labeled using DIG-11-dUTP in a single-cycle PCR. In colony blot hybridization, the probes were specific for the insert DNA from which the probe was derived and, importantly, did not detect vector sequences.

INTRODUCTION

The production of probes from the insert DNA of cloned cosmids or plasmids is a frequent requirement in molecular biology. Probes are commonly made by nick-translation (13) or by random-primed labeling (4). However, these techniques label the entire template DNA, including, if present, vector sequences. Production of vector-free probes therefore normally requires restriction enzyme digestion of the DNA, agarose gel electrophoresis, excision of the desired insert DNA, then labeling by one of these methods. This procedure fails for cosmids libraries from which insert DNA cannot be re-isolated by virtue of the cloning site having been lost. Moreover, if the insert DNA is large, its recovery might be inefficient or require the excision of several bands from an agarose gel. DNA can also be labeled by nick-translation followed by addition of excess vector DNA; vector and insert DNA can then be separated by hydroxylapatite chromatography under appropriate conditions (5). These procedures, even when applicable, are extremely time-consuming and vary from probe-to-probe sequence. Other techniques involving polymerase chain reaction (PCR) labeling of insert DNA (e.g., Reference 12) usually require knowledge of sequence in the insert, and separate primers are required for each insert tested.

We have constructed a library of genomic DNA of *Salmonella typhimurium* in the cosmid vector pHC79 (14). The insert DNA is on average 40 kb in length, which is too long for high-fidelity amplification by PCR in one piece, and the restriction site in the vector used to clone the genomic DNA was lost during cloning. Our requirement was the reproducible production of probes from insert DNA from a large number of different clones using vector-specific primers that did not detect vector sequences. This has led us to devise a new approach to the production of insert-specific probes, based on the following rationale.

Probe DNA was produced separately from each end of insert DNA. Since we were interested in detecting overlapping clones, and all insert sequences were approximately the same size, two probes (each specific for one end) used as a pair can be regarded as equivalent to a probe derived from the full-length insert DNA. End-specific probes were constructed from small derivatives as follows. Digestion of the cloned DNA with a restriction enzyme with a 6-bp recognition site cleaved the DNA at a single site in the vector DNA (in our case, *Eco*RI or *Sal*I; see Figure 1) and potentially at one or several sites in the insert DNA, the positions of which will vary between clones. Ligation of the restriction fragments at low concentration, which favors re-circularization over concatamerization, produced a mixture of circular molecules. One of these contained most of the vector and a region of insert DNA significantly smaller than 40 kb, which could serve as a template for an insert-specific PCR product. PCR using vector-specific primers and subsequent digoxigenin (Dig)-labeling of the PCR product yielded a labeled probe from one end of the insert DNA. The process was repeated using the second restriction enzyme to produce a probe from the other end of the insert DNA.

MATERIALS AND METHODS

Bacterial Strains and Cosmids

*A. typhimurium* genomic library (14) was used for the purpose of evaluating the method. This consists of DNA from *A. typhimurium* TML (6) cloned into a cosmid vector (pHC79; Reference 8) in *A. typhimurium* LT7 (10). Organisms are plated onto L Agar (10 g trypotene, 5 g yeast extract, 10 g NaCl and 15 g agar/L) and recombinant clones selected by carbenicillin resistance (250 mg/mL Pyopen™; Link Laboratories Ltd., Glasgow, Scotland, UK). *Escherichia coli* HB101 (recA13) (2) is used for routine molecular biological manipulations.
Short Technical Reports

Table 1. Primers Used in PCR

<table>
<thead>
<tr>
<th>Primer Number</th>
<th>Sequence 5′–3′</th>
<th>PCR</th>
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</thead>
<tbody>
<tr>
<td>P1</td>
<td>GTCCGCGTAGAGGATC</td>
<td>Standard, EcoRI template</td>
</tr>
<tr>
<td>P2</td>
<td>GCCCTTTCTGCTTTCAAGAATTC</td>
<td>Standard, EcoRI template</td>
</tr>
<tr>
<td>P3</td>
<td>CACCGTGCTGTTGGATC</td>
<td>Standard, Sall template</td>
</tr>
<tr>
<td>P4</td>
<td>CAAGGGCATCGGTGGATC</td>
<td>Standard, Sall template</td>
</tr>
<tr>
<td>P5</td>
<td>CGATGCCGCGGCGTGAAGGATC</td>
<td>Long, EcoRI template</td>
</tr>
<tr>
<td>P6</td>
<td>GTATCAGGAGGCCCTTCCTTCAAGAATTC</td>
<td>Long, EcoRI template</td>
</tr>
<tr>
<td>P7</td>
<td>CGACACGACCCGCTGCTGGATC</td>
<td>Long, Sall template</td>
</tr>
<tr>
<td>P8</td>
<td>GGCTCTCAAGGGGATCGCGTGCAC</td>
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</tr>
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Primers were designed from the vector sequence flanking the insert DNA. Primers for the standard PCR (P1–P4) have a calculated annealing temperature of 56°C, and primers for the Long PCR (P5–P8) have a calculated annealing temperature of 65°C.

Transformation Methods

Preparation of competent *E. coli* is performed using rubidium chloride buffers (7). Cosmids are transformed into *S. typhimurium* LT7 by electroporation (3).

Preparation of Template DNA

Cosmid DNA is isolated from the clone of interest by standard rapid small-scale alkaline lysis (16), and 0.5 µg is cleaved with *Eco*RI or *Sal*I (4 U, 1 h; Life Technologies, Paisley, Scotland, UK), extracted with phenol/chloroform and precipitated with ethanol (16). Dilutions containing 100, 10 or 1 ng of digested DNA are ligated using 0.5 U T4 DNA Ligase (Life Technologies) for 2 h at 37°C in a total volume of 20 µL. All restriction enzymes and DNA modifying enzymes are used as recommended by the manufacturers and in the buffers supplied.

PCR

Primers (P1–P8; Table 1) were constructed by Alta Biosciences, University of Birmingham, UK. Figure 1 shows the positions of the primers on the vector sequence. PCR was performed as follows.

Standard PCR with short templates. Approximately 0.25 ng of the template DNA is amplified in a 50-µL reaction mixture containing 400 nM of each forward and reverse primer (primer pairs 1 and 2 or 3 and 4), 200 µM of each dATP, dCTP, dTTP and dGTP

Figure 1. Construction of deletion derivatives from pHC20·2 for use as templates in PCR. The positions of the relevant restriction sites in the pH79 vector (dark box) are indicated, and *Eco*RI and *Sal*I restriction sites are shown in the insert DNA of the parent cosmid pH20·2. Restriction digestion with *Eco*RI followed by ligation results in the mixture pH20·2/*Eco*RI, which includes cosmid pH20E. Digestion with *Sal*I followed by ligation results in the mixture pH20·2/*Sal*I, which includes cosmid pH20S. The positions of the primers (P1–P8) used in PCR are indicated by black bars.

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(Boehringer Mannheim, Lewes, East Sussex, England, UK), 1.25 U of Taq DNA Polymerase (Qiagen, Crawley, West Sussex, England, UK), 1× reaction buffer (supplied by the manufacturer) and 20% of Qiagen Q solution PCR optimization buffer. Reactions are performed in 0.5-mL tubes on a PTC-100™ Thermal Cycler (MJ Research, Watertown, MA, USA) with a heated lid in place. Following an initial denaturation step (94°C for 1 min), 30 PCR cycles are carried out, each consisting of 94°C for 30 s, 56°C for 30 s, an extension time of 5 min at 72°C with one final extension of 30 min at 72°C. A 5-µL aliquot of the amplified DNA is analyzed by agarose gel (0.7%) electrophoresis.

**PCR with long templates.** Approximately 0.25 ng of ligated DNA is amplified in a total reaction volume of 50 µL. The reaction mixture also contains 300 nM of each forward and reverse primer (primer pairs 5 and 6 or 7 and 8), 500 µM of each dATP, dCTP, dTTP and dGTP, 2.6 U of combined Taq and Pwo DNA Polymerases (Expand™ Long Template PCR System; Boehringer Mannheim) and 1× Reaction Buffer 3 (supplied by the manufacturer). Reactions are performed in 200-µL tubes, overlaid with 30 µL mineral oil and cycled without using a heated lid (a UNO-Thermoblock™ machine [Biometra GmbH, Gottingen, Germany] was used in this instance, but the machine and model is unlikely to be significant). After an initial denaturation step (92°C for 2 min), 10 cycles are carried out, each consisting of 92°C for 10 s, 63°C for 30 s and 68°C for 360 s. An additional 20 cycles are carried out in which the extension time of each cycle is extended by 20 s at each cycle. This is followed by a final extension of 7 min at 68°C. An aliquot (5 µL) of each PCR product was analyzed by agarose gel electrophoresis.

**Incorporation of DIG-11-dUTP into PCR Product**

One-fifth of the PCR product (10 µL, containing 60–100 ng of DNA) is taken and added to a new reaction mixture.

**For short PCR products.** A total volume of 20 µL containing 2 µL of DNA Dig-labeling mixture (1:2 DIG dUTP:dTTP; Boehringer Mannheim), 1× PCR buffer and a further 1 U Taq DNA Polymerase (Qiagen) are used. A single cycle of PCR is performed: 94°C for 1 min, 56°C for 30 s and 72°C for 10 min.

**For long PCR products.** A total volume of 20 µL containing 2 µL of DNA Dig-labeling mixture (1:2 DIG dUTP:dTTP), 1× PCR Buffer 3 and a further 1 U of Expand Long Template Polymerase are used. The reaction is overlaid with mineral oil, and one cycle is performed: 92°C for 2 min, 63°C for 30 s and 68°C for 20 min.
During all amplification procedures, precautions were taken to minimize the risk of cross-contamination, including use of Biosphere aerosol-resistant pipet tips (Sarstedt, Leicester, England, UK).

Quantitation of Dig-Labeled PCR Products

A series of tenfold dilutions (to $10^{-5}$) of each probe (1 µL) is spotted onto nylon membranes, along with 1 µL of tenfold dilutions of control labeled DNA (both from Boehringer Mannheim). The membranes are analyzed directly using a Chemiluminescence Detection Kit (Boehringer Mannheim).

Production of Vector-Specific Probe

pHC79 DNA (1 µg) is labeled by random priming (4) using DIG DNA Labeling Mixture, random hexanucleotides (both from Boehringer Mannheim) and DNA Polymerase I, Klenow Fragment (Life Technologies) (1).

Hybridization, Southern Blots and Colony Lifts

PCR products (5 µL) and DIG-labeled $\lambda$HindIII Markers (5 µL; Boehringer Mannheim) are separated by electrophoresis on a 0.7% agarose gel and immobilized on positively charged nylon membranes by Southern blot transfer followed by UV irradiation (1).

Colony lifts are made using Nylon Membranes for Colony and Plaque Hybridization (Boehringer Mannheim), and DNA is immobilized by UV irradiation. The membranes are then treated with Proteinase K (Boehringer Mannheim) to remove excess cell debris (1).

Filters are prehybridized for 1 h in hybridization buffer (5× SSC [0.75 M NaCl, 75 mM sodium citrate, pH 7.0], 50% formamide [Merck, Lutterworth, Leicestershire, England, UK], 0.1% sodium lauryl sarcosine, 0.02% sodium dodecyl sulfate [SDS], 2% Blocking Reagent [Boehringer Mannheim]). Membranes with immobilized PCR products are hybridized with 1.5 µg of the vector-specific probe, and the colony lifts are hybridized with 20 ng of the labeled PCR product per mL of hybridization solution. Hybridizations are carried out at 42°C overnight. Membranes are washed to remove nonspecifically bound probe, and positive clones are detected using the DIG Luminescent Detection Kit (1) followed by exposure to X-ray film (Hyperfilm™ MP; Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) for periods determined by the sensitivity of the probe.

RESULTS AND DISCUSSION

The method was evaluated using a
single clone (pHC20·2) from our library, which had previously been extensively characterized and mapped by restriction analysis, which made it possible to construct two deletion derivatives of pHC20·2 to act as controls in our validation experiments.

Evaluation of the Method

DNA from the cosmid pHC20·2 was cut with EcoRI; only one EcoRI restriction site is present in the vector DNA, approximately 375 bp from the BamHI cloning site, and insert DNA contained several EcoRI cleavage sites (Figure 1). With pHC20·2, restriction digestion with EcoRI and re-circularization resulted in a mixture of molecules (pHC20·2/EcoRI), which included a cosmid with a 5-kb insert (pHC20E), the only product containing the primer binding sites and capable of replication. A small fragment (375 bp) of vector (EcoRI to BamHI sites) was lost from this cosmid (Figure 1). pHC20E was selected by standard transformation of the ligated DNA into E. coli, re-purification and transformation into S. typhimurium LT7 by electroporation to provide a “validation” control.

Similarly, restriction digestion with SalI and re-circularization resulted in a mixture of molecules (pHC20·2/SalI), which included a cosmid (pHC20S) with a 6-kb insert from which a small fragment of vector (SalI to BamHI sites) was lost (Figure 1). pHC20S was selected as for pHC20E. The EcoRI and SalI restriction sites in pHC79 are positioned such that the origin of replication was included in pHC20E and pHC20S. The construction of these control cosmids was important for validation but is not required for the application of this method.

Approximately 0.25 ng of each of the ligations of pHC20·2/EcoRI DNA and the control pHC20E were used in standard PCR with Primers 1 and 2, yielding products of the expected size (5 kb) from both.

pHC20·2/SalI and pHC20S DNA were amplified using the PCR method for long (>5 kb) template using Primers 7 and 8. Products of the expected size (6 kb) were achieved with pHC20S and the ligations using 100 and 10 ng of restriction-digested DNA. A product was not obtained from the ligation using 1 ng of restriction-digested DNA; the excess magnesium ions carried across from this ligation is thought to have inhibited PCR with this polymerase.

Single PCR products were produced; when very long templates were used, faint nonspecific bands were seen. The PCR products were tested for the presence of products containing vector sequences by Southern hybridization. Several bands that contained the vector sequence were found.
in the PCR products from the 100 and 10 ng ligations, but not found in products with 1-ng ligations of pH20·2/EcoRI DNA. At the higher concentrations it is likely that re-ligation of fragments to each other instead of re-circularization, coupled with nonspecific annealing of the primers, would mean that the vector could be incorporated into nonspecific PCR products. Ligation using 1 ng of restriction-digested DNA favors re-circularization over concatamerization, and stringent PCR conditions such as those used above minimizes nonspecific priming. Non-specific PCR products containing vector sequences were not found in either the 100- or 10-ng ligations using pH20·2/SalI DNA; highly stringent reaction conditions were used during amplification with the Expand Long Template Polymerase.

The PCR products that did not contain vector DNA were labeled with DIG-11-dUTP in a further cycle of PCR as described in Materials and Methods. The Dig-labeled PCR products were tested for specificity by colony hybridization. A colony lift was prepared from: (i) clones containing pH20·2 insert DNA, (ii) clones that were known to contain different insert DNAs and (iii) clones that contained only vector DNA. The labeled PCR product (at 20 ng/mL) from the 1-ng ligation of pH20·2/EcoRI DNA was used in colony hybridization and detected only those clones that contained pH20·2; it did not detect any clones containing different-insert DNA or vector-only DNA (Figure 2). A similar result was obtained using the labeled PCR product from the 10-ng ligation of pH20·2/SalI DNA as template (data not shown).

Clearly, if desired, the long PCR method could be used throughout or used when standard PCR cannot (for whatever reason) be used reliably to amplify templates <5 kb.

Application of this Method to Other Members of the Cosmid Library

This method has been applied successfully to 10 additional clones from the library. In our hands, PCR products <5 kb can be successfully obtained by standard PCR using primers 1–4. Modified clones containing longer inserts (5–10 kb) were amplified using Expand Long Template Polymerase and primers 5–8. Probes have been derived from both ends of the insert DNA in these 10 clones, and the probes did not detect vector sequences, as demonstrated by colony lift hybridization. These probes was efficiently labeled, and 10–500 ng/μL of Dig-labeled DNA were obtained from the PCR product.

This method of producing probes separately from each end of insert DNA overcomes the problems inherent in selectively labeling large fragments of DNA. Endonuclease digestion, ligation and stringent PCR followed by Dig-labeling produced probes from each end of the insert DNA that did not detect vector sequences. Dig-labeling of the PCR products in a subsequent cycle of PCR significantly reduced the amount of time needed for the production of the probe. Incorporation of Dig during 30-cycle PCRs would have reduced the

Figure 2. Specificity of probes in colony hybridization. An autoradiograph of a membrane hybridized with luminescent probe made from pH20·2/EcoRI. The membrane was set up as follows: (A) a row of six clones that contain vector DNA only; (B) a row of six clones that contain DNA from pH20·2; (C) six rows of clones, containing four clones of pH20·2 DNA.
rate of amplification and the fidelity and yield of the product because the DIG-11-dUTP is not incorporated as efficiently as unlabeled nucleotides by the polymerase (15). Single-step Dig-labeling works well when amplifying short fragments (<1 kb), but inefficient Dig incorporation necessitates long PCR times for large fragments. It has also been recommended to Dig-label PCR products during an additional 30 cycles of PCR (11), often using nested primers (e.g., Reference 9). We have demonstrated that incorporation of DIG-11-dUTP during one further cycle of PCR produces suitable probes.

Probes produced by this method can be used sequentially or together as long as the Dig-labeled PCR products have been quantified as described in Materials and Methods and are used at the same concentration in hybridization. We foresee that these probes will have many applications in addition to screening a cosmid library, for example, in Southern hybridization against chromosomal DNA (our unpublished data).

REFERENCES


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