Benchmarks

Direct Primer Walking on P1 Plasmid DNA

BioTechniques 23:98-100 (July 1997)

The development of vectors such as P1, P1-derived artificial chromosome (PAC) or bacterial artificial chromosome (BAC) (2,6,8) has permitted the cloning of large DNA fragments. These vectors have become common in large-scale sequencing and mapping projects. Therefore, a reliable primer walking protocol for P1 plasmids is desirable.

It has been demonstrated previously that sequencing of P1 plasmid DNA is generally feasible (4,11). However, the protocols included several laborious steps such as a host strain change from NS3145 to DH10B or tedious purification procedures and were limited to the use of primers located in the vector arms. Another development for sequencing P1 plasmid DNA uses a polymerase chain reaction (PCR)-based technique termed “thermal asymmetric interlaced PCR” (5); however, this method also relies on numerous experimental steps. We present an optimized sequencing protocol for direct primer walking on P1 plasmid DNA based on cycle sequencing (7).

The effect of the following parameters on the success rate of P1 sequencing was evaluated: host cell type, purification procedure, template amount, primer design, primer amount and cycle sequencing conditions. Sequencing reactions were carried out with either fluorescein isothiocyanate (FITC)- or Cy5™-labeled primers and analyzed on the corresponding sequencing apparatus (ALF DNA Sequencer™ and ALF-express™; Pharmacia Biotech, Piscataway, NJ, USA). The read length assigned to sequencing results was determined by the number of nucleotides called after automatic processing by the ALF software.

P1 plasmid DNA was isolated from its E. coli host strain NS3145 (library strain) and transformed into the new host strain DH10B by electroporation (3). When performing parallel P1 plasmid DNA preparations under identical conditions (2× YT medium, 0.5-L culture volume, 50 µg/mL kanamycin, single-colony inoculate, two QIAGEN® 100 columns [Qiagen, Hilden, Germany]), the E. coli host strain NS3145 consistently yielded between 5 and 8 times more P1 plasmid DNA than the DH10B host strain. On average, a 0.5-L culture (NS3145 strain) yielded 80 µg (±10%) plasmid DNA.

P1 plasmid DNA purification was performed with a conventional alkaline lysis/phenol:chloroform protocol (1) (Nucleobond®, Macheray and Nagel, Düren, Germany) or an alkaline lysis protocol followed by column purification (Qiagen). The Nucleobond and the Qiagen protocols provided by the suppliers were both modified as follows. Lysis time was shortened from 5 to 1 min, and volumes of the resuspension, lysis and neutralization buffers were

Figure 1. Direct P1 plasmid sequencing using 6 pmol of an FITC-labeled primer (20-mer) and 8 µg of column-purified P1 plasmid DNA.
doubled. Elution was done with pre-
heated buffer (55°C) and a volume in-
creased by one fourth. In both cases,
the precipitated P1 plasmid DNA was
rinsed twice with 70% ethanol. Care
was taken not to overdry the pellet after
the final wash. Multiple sequencing re-
actions were performed on plasmid
DNA grown in either NS3145 or
DH10B (ELECTROMAX™; Life Tech-
nologies, Berlin, Germany) and isolat-
ed by both methods.

DNA purified from the host strain
NS3145 yielded better sequencing re-
results than identical amounts from the
host strain DH10B. Although plasmid
DNA isolated by conventional alkaline
lysis/phenol:chloroform purification
yielded usable sequence, column puri-
fication reduced the background in the
sequencing reactions and produced
longer readouts with fewer ambiguities.

Best results were obtained when 5–10 µg of plasmid DNA were used in
the reaction. Thus, substantially larger amounts of DNA are necessary for suc-
sessful cycle sequencing of P1 clones than of smaller multicopy plasmids.

Walking primers were selected and
designed with the aid of the European
Molecular Biology Laboratory (EMBL)
integrated software package Gene-
Skipper (9). In general, primers were
designed by choosing a region with the
highest possible melting temperature
(calculated melting temperatures were
above 45°C). This approach makes it
possible to use a high annealing temper-
atures during cycle sequencing and thus
to reduce nonspecific priming events.
Primers either 20 or 27 nucleotides in
length were tested. There was no note-
worthy difference in their performance.
Walking primers were placed an aver-
age of 40 bases away from the end of
the sequence determined by the previ-
uously used primer.

The optimal amount of primer was
found to be 6 pmol with a range of
4–10 pmol per reaction giving good re-
results. Too high an amount of primer led
to nonspecific priming, which rendered
sequence interpretation difficult. Best
results were achieved with a two-step
cycling protocol as follows: (i) initial
denaturation at 95°C for 3 min and (ii)
40 cycles at 60°C for 30 s and 95°C for
30 s. In general, the commercially
available Thermo Sequenase™ Kit
(Amersham International plc, Cam-
bridge, England, UK) (10) was used.
However, other enzymes performed
equally well (data not shown).

Applying the above protocol, 44
primer walks on various P1 clones have
been performed, and almost 17 kb of
sequence have been determined. The
average reading length per sequencing
reaction was 385 bases using 30-cm
glass plates. The estimated sizes of the
clones (about 95 kb) were well within
the range of average P1 clones. The
above protocol has been applied to
BAC clones with equal success. Table 1
gives an overview of all walks per-
formed on P1 plasmid DNA, and Figure
1 shows a typical sequencing reaction.

In summary, a bacterial host strain
change is superfluous when P1 plasmid
DNA for sequencing is prepared, be-
cause DNA isolated from the NS3145
strain performs well in cycle sequenc-
ing. The best results were obtained us-
using 5–10 µg of P1 plasmid DNA and 6
pmol of fluorescently labeled primer in
combination with a two-step cycle se-
quencing protocol with a high anneal-
ing temperature (60°C). Column-puri-
fied DNA performed better than DNA
isolated by alkaline lysis only.

Note Added in Proof: We recently
found that a cycling protocol consisting
of an initial denaturation at 95°C for 3
min and 40 cycles at 60°C for 30 s,
68°C for 30 s and 95°C for 30 s further
improves the average length read.

REFERENCES

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combinant plasmid DNA. Nucleic Acids Res.
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Table 1. Overview of Walks Performed on P1 Plasmid DNA

<table>
<thead>
<tr>
<th>Clone Number</th>
<th>Number of Primers Used</th>
<th>Bases Read in Each Sequencing Reaction</th>
<th>Number of Failed Sequencing Reactions</th>
<th>Average Length Read</th>
<th>Total Bases Sequenced</th>
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<tr>
<td>3A</td>
<td>11</td>
<td>253, 345, 423, 253, 487, 253, 418, 578, 405, 487, 337</td>
<td>0</td>
<td>385 bases</td>
<td>4239 bases</td>
</tr>
<tr>
<td>6A</td>
<td>10</td>
<td>323, 271, 100, 251, 0, 334, 266, 549, 505, 508</td>
<td>1</td>
<td>311 bases</td>
<td>3107 bases</td>
</tr>
<tr>
<td>12B</td>
<td>23</td>
<td>534, 279, 360, 329, 384, 239, 194, 273, 149, 441, 619, 408, 476, 413, 348, 633, 168, 462, 448, 649, 513, 661, 617</td>
<td>0</td>
<td>417 bases</td>
<td>9597 bases</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td></td>
<td>1</td>
<td>385 bases</td>
<td>16943 bases</td>
</tr>
</tbody>
</table>

A total of 44 primers were used on three different P1 clones. A total of 16943 bases were sequenced with an overall average of 385 bases read per sequencing reaction. One primer gave no sequence.
Fabrication of Ion-Selective Microelectrodes by a Centrifugation/Suction Method

Biotecniques 23:100-102 (July 1997)

The preparation of ion-selective microelectrodes can be both time-consuming and frustrating. Since most techniques involve filling the tip with resin before back-filling the rest of the electrode with electrolyte, the step most commonly found annoying is the elimination of air bubbles between the electrolyte and the resin. Since the tip is not visible, trying to draw liquid ion exchanger (LIX) into a plugged electrode tip is also frustrating. In fabricating calcium ion-sensitive microelectrodes for use as extracellular vibrating probes, Kühbreiber and Jaffe (2) avoided these problems by sequential suction-front-filling, first with electrolyte and then with resin. Although these steps precluded the problem of air bubbles between the two filling materials, the electrodes described had open tips of 1–10 µm. Intracellular ion-selective electrodes require smaller tip sizes, and front-filling the electrolyte becomes inconvenient (1) or impossible. Back-filling the tip through the capillary action of a filling fiber (3,5) is undoubtedly the most common practice today for preparing standard potassium chloride electrodes, but due to the hydrophobicity of the coating, silane-coated micropipets cannot be filled in this manner. Yet, if the problem of fully filling the electrode tip with electrolyte can be overcome, the advantage of subsequent bubble-free front-filling with LIX can be achieved. I describe a simple technique by which this may be accomplished and that nearly always results in a useful ion-selective electrode.

First, electrodes are batch-silane-coated. Depending on the method of silane coating employed, capillary glass tubing may be either with or without filling fibers. The technique I use is based on procedures described by Spray and Zavilowitz (4) for double-barreled electrodes, but works well with single-barreled electrodes with a filling fiber. One part trimethylchlorosilane (silane) (Sigma Chemical, St. Louis, MO, USA) is mixed with 10 parts carbon tetrachloride. A small amount of silane is introduced into the back of the electrode and allowed to move into the tip. Because of the filling fiber and the low surface tension of the solution, the silane travels easily to the tip. Excess solution is then withdrawn, and the electrodes are placed horizontally in a carrier made from an aluminum block and baked in an oven at 110°C for 2 h.

The excellent synthesis of fluorescently labeled primers by Heiko Drzonek and Walter Schartau as well as the fine technical support by Karin Bauer are gratefully acknowledged. Financial support came from the DFG. Address correspondence to Christian Kilger, Institute of Zoology, University of Munich, P.O. Box 202126, D-80021 Munich, Germany. Internet: kilger@zi.biologie.uni-muenchen.de

Received 7 August 1996; accepted 24 January 1997.

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