Generation of Genomic Mini-Libraries by Taq DNA Polymerase Modification of Genomic Fragments


When cloning genomic DNA fragments, the efficiency is greatly reduced as the size of the restriction fragment increases. Libraries derived from the total genome are therefore biased because shorter fragments will be cloned preferentially. When cloning a particular gene, it is most desirable to determine a restriction enzyme that will generate a fragment of a reasonable size for the gene of interest by Southern blotting. A mini-library can then be created containing only the genomic fragment of that particular size and reducing the number of colonies that have to be screened. This procedure will inevitably mean that a different restriction enzyme is used for each fragment cloned, and therefore a fresh batch of digested, phosphatased vector must be prepared each time. The digestion and dephosphorylation reactions must both be highly efficient to obtain a high percentage of insert uptake, and often this is the rate-limiting step in mini-library creation. We have circumvented this problem by using a commercially available vector designed for the cloning of polymerase chain reaction (PCR) products. The pGEM®-T Vector (Promega, Madison, WI, USA) contains a single 3′ T overhang that allows the insertion of PCR products with overhanging A residues added by the terminal transferase activity of certain Taq DNA polymerases that lack proofreading activity (1,2). Taq DNA polymerase can be used to create similar overhanging ends on any blunt-ended fragment, and these can then be efficiently cloned into pGEM-T, which gives a low background of recircularization without insert. This method also increases the range of restriction enzymes that can be screened to identify a fragment of the desired size because it is not limited by those present in vector multiple cloning sites. It is equally useful if two restriction enzymes are used to generate a fragment of the desired size.

This method is particularly useful in isolating clones from organisms whose DNA is of an unusual nucleotide content and so tends to be unstable when propagated in bacteria. For example, the genomes of Dictyostelium discoideum and Malaria plasmodium are both extremely AT-rich and notoriously unstable, especially in high-copy-number vectors. This means that it is impractical to amplify a genomic library for repeated use because the amplification procedure leads to loss and rearrangement of a significant percentage of the clones. Thus, even if many genes of interest can in theory be isolated from the same library, in practice it is necessary to regularly remake the library to avoid amplification steps.

We have used this system to clone a 3-kb genomic fragment containing the mo15 gene from the cellular slime mold D. discoideum. Genomic clones were identified by hybridization to a partial cDNA clone from Dictyostelium encoding a protein highly homologous to Xenopus cyclin-dependent protein kinase MO15 (cdk7) (3). As stated above, isolating genomic clones from this particular organism can be problematic because the extremely AT-rich nature of the DNA makes many fragments unstable for cloning into high-copy-number vectors. We have made several unsuccessful attempts to isolate genomic clones encoding mo15 from conventional Dictyostelium libraries that had successfully been used to isolate other genes.

**Figure 1.** Average insert size in genomic mini-library. Six white colonies were picked at random and the plasmid DNA isolated using the Wizard® purification procedure (Promega). The DNA was digested with PvuII, which removes the insert and 500 bp of flanking vector DNA, and resolved by electrophoresis on a 0.8% agarose gel. The Ladder Mix (MBI Fermentas Ltd., Vilnius, Lithuania) was run to allow size determination (M). The vector (2567 bp) and a single insert band are seen in all six cases.

Cloning of fragments into pGEM-T. The purified fragments were incubated with 25 mM dNTPs and 5 U of Taq DNA polymerase (Boehringer Mannheim, Lewes, East Sussex, England, UK) in the buffer recommended by the manufacturer at 72°C for 30 min. The polymerase activity of the Taq enzyme created blunt-ended fragments, and then the terminal transferase activity caused the addition of A residues to the 3′ ends of the blunt fragments. After cleaning with GENE CLEAN II, the fragments were ligated with 25 ng of pGEM-T at 4°C for 18 h in a 10-µL ligation mixture. This mixture (2.5 µL) was transfected into UltraMAX DH5α™ Competent Cells (Life Technologies, Paisley, Scotland, UK) by a chemical transformation method and 10% of the cells plated onto ampicillin (100 µg/mL) resistance plates containing 80 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to allow for blue/white selection of the colonies with inserts. The percentage of white colonies containing insert was dependent on the amount of genomic DNA originally present in the restriction digest. In this instance, starting with 60 µg of genomic DNA rather than 20 µg led to a 9-fold increase in the number of white colo-
Benchmarks

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Small-Scale Preparation of the Single-Copy Bacterial Artificial Chromosome Vector pBeloBAC11


The construction of stable, large-insert DNA libraries is critical for the analysis of complex genomes (8). Traditionally, yeast artificial chromosomes (YACs) (1) have been the system of choice, but this system suffers from a number of disadvantages. YAC libraries can be difficult and tedious to construct, cloned sequences are often unstable or

REFERENCES


Isolation of the mo15 gene. Colonies derived from the the most efficient insertion ligation were screened using the mo15 cDNA clone as probe (3), and out of approximately 8000 white colonies screened, 20 came through two rounds of screening containing the mo15 gene. This was confirmed by PCR analysis using primers based on the known coding sequence (data not shown). When DNA was isolated from two of these, the insert size was found to be approximately 3 kb, as expected, and DNA sequence analysis confirmed the presence of the mo15 gene. These are independent isolates because restriction analysis revealed that each was in a different orientation relative to the vector sequences (data not shown).

This quick and efficient method for mini-library creation increases the range of restriction enzymes that can be used to isolate a fragment of suitable size and circumvents the necessity to generate a batch of digested, phosphatased vector with a low background of self-ligation for each restriction enzyme used. It is particularly useful when isolating clones from organisms whose DNA is unstable in bacteria, because the desire not to amplify libraries means that it is often necessary to generate a fresh library several times to isolate more than one gene.