Plasmid Construction by Linker-Assisted Homologous Recombination in Yeast

BioTechniques 31:1246-1250 (December 2001)

Homologous recombination of DNA in yeast can be used to construct recombinant plasmids with no need for specific restriction sites (7,9,12). This is most rapid and convenient when the DNA substrates for homologous recombination can be made in a single step by PCR amplification (5,6,8,10). For applications in which PCR amplification is difficult or undesirable, “linker-assisted” homologous recombination is an alternative that avoids the need for PCR amplification of the DNA substrates by using dsDNA linkers to guide recombination (2,11). These methods are applicable when a sequence-verified plasmid clone is available but further PCR amplification is undesired. However, the reported methods are complicated by the need for large oligonucleotides. Furthermore, individual recombinant clones could not be identified phenotypically. Here we describe a novel method for making the double-stranded linkers and a counter-screen that can be used to help identify which yeast transformants contain exclusively the recombinant plasmid. In addition, this method allows the use of a circular plasmid substrate.

To illustrate the method, we recombined the GFPuv gene (BD Biosciences Clontech, Palo Alto, CA, USA) into a yeast expression vector containing the yeast TRP1 gene in its multiple cloning site. Yeast transformants containing recombinant plasmids could then be identified directly by green fluorescence and indirectly by tryptophan auxotrophy. There are two steps in this procedure: (i) production of the double-stranded linkers (Figure 1) and (ii) homologous recombination during transformation of yeast (Figure 2). Making the double-stranded linkers involved four oligonucleotides that were used to prime PCR amplification of small segments of DNA flanking the multiple cloning site of vector YEp195AC (Figure 1). This produced two 240-bp dsDNA linkers, consisting of 200 bp corresponding to the DNA sequence of the vector flanking the cloning sites and 40 bp corresponding to the ends of the GFP open reading frame. Note that the 40-bp segments corresponding to GFP are derived exclusively from the sequence of the original oligonucleotides. In principle, the double-stranded linkers can be made by PCR amplification of any plasmid that has the same 200-bp flanking sequences as the intended cloning vector,
including the cloning vector itself. In this case, we used a plasmid with a different yeast selectable marker, URA3, to eliminate the possibility that it could be selected in the subsequent yeast transformation, in which LEU2 transformants were selected.

The double-stranded linkers were used to direct homologous recombination between a circular plasmid carrying GFPuv and a linearized plasmid, YEp181AC-TRP1, prepared by restriction digestion with PsII and Sall (Figure 2). The restriction digestion with these two enzymes greatly reduced the number of non-recombinant plasmids subsequently obtained (not shown). Note that four different DNA molecules are involved in the homologous recombination during transformation of yeast. Leu+ transformants were selected by growth on medium lacking leucine, then scored for GFP expression by fluorescence under ultraviolet light, and scored for growth in the absence of tryptophan by replica plating (Table 1A). Of the 122 transformants obtained, 25 contained the ADH-GFP recombinant allele, as inferred from their fluorescence on glucose medium. 8 of the colonies were both fluorescent and Trp+, as expected for yeast transformants that contain both recombinant and non-recombinant plasmids. All of the 17 Trp- transformants were fluorescent, showing that the absence of the TRP1 marker was a useful indication of which transformants contained recombinant ADH-GFP plasmids. A plasmid from each of 12 such Trp- transformants was recovered in E. coli, and the DNA sequence of each was verified; all 12 had exactly the correct recombinant sequence. To compare the use of circular donor plasmid with linearized donor plasmid, the donor plasmid was digested with the restriction enzyme EcoRI before transformation.

Table 1. Identification of Yeast Transformants Containing ADH-GFP Recombinant Plasmids

<table>
<thead>
<tr>
<th>(A) Circular Donor Plasmid</th>
<th>(B) Linear Donor Plasmid</th>
<th>(C) Linkers Omitted</th>
</tr>
</thead>
<tbody>
<tr>
<td>phenotype</td>
<td>GFP+</td>
<td>GFP</td>
</tr>
<tr>
<td>Trp+</td>
<td>8</td>
<td>97</td>
</tr>
<tr>
<td>Trp-</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

(A) Yeast strain W303 (ATCC, Manassas, VA, USA) was transformed with the following amounts of the four pieces of DNA illustrated in Figure 2: 100 ng YEp181AC-TRP1 (digested SalI/PstI), 1 µg pYES2-GFP (circular), and 0.5 µg each of the double-stranded linkers. Leu+ transformants were selected and scored for fluorescence (GFP+) and tryptophan auxotrophy (Trp+). Fluorescence on glucose medium indicates that the yeast clone contains the recombinant ADH-GFP allele. Trp- indicates that the yeast transformant most likely contains exclusively the recombinant plasmid.

(B) The same experiment was carried out except that the plasmid pYES2-GFP was linearized by digestion with the restriction enzyme EcoRI before transformation.

(C) The same experiment was carried out, except the DNA linkers were omitted.

Figure 2. Linker-assisted recombination and counterselection in yeast. The double-stranded linkers guide recombination between the donor and acceptor plasmids, as indicated (not shown to scale). The acceptor plasmid, YEp181AC-TRP1, was made by ligating a BamHI fragment containing the TRP1 gene from plasmid YdpW (1) into the BamHI site of YEp181AC (4) and was digested with the restriction enzymes PsII and Sall before use, as indicated by an arrow. The donor plasmid, pYES2-GFPuv, was made by ligation of the GFPuv gene (BD Biosciences Clontech) into the vector pYES2 (Invitrogen, Carlsbad, CA, USA). This vector causes expression of GFP only in the presence of galactose. Recombination to obtain the ADH-GFP allele, in vector YEp181AC-GFP, occurred when a mixture of these four DNAs was used for transformation of yeast strain W303 (ATCC) in the presence of lithium acetate (3) and could be detected by expression of GFP on glucose medium.
tion enzyme EcoRI before the transformation of yeast (Table 1B). An increased number, 43, of the transformants were recombinants. When the DNA linkers were omitted, fewer transformants and no recombinants were obtained (Table 1C).

In further cloning experiments using other genes (not shown), we have found that the indirect method of detection of recombinants, by loss of TRP1, is a useful method to help identify yeast transformants that contain exclusively recombinant plasmids. In each case, the DNA sequence of clones was confirmed directly. For cloning of DNA fragments that did not have a phenotype such as fluorescence, this greatly reduced the labor required to obtain recombinant plasmids. This method is particularly applicable when the end product is a yeast expression vector because it eliminates conventional cloning steps in bacteria, minimizes PCR amplification errors in the cloned DNA, and does not require unique restriction sites in the donor plasmid substrate.

REFERENCES

Address correspondence to Dr. Jeremy H. Toyn, Experimental Station, E400/3227, DuPont Pharmaceuticals Company, Wilmington, DE 19880, USA. e-mail: jeremy.h.toyn@dupontpharma.com

Received 15 May 2001; accepted 5 September 2001.
Paul L. Gunyuzlu, Gregory F. Hollis, and Jeremy H. Toyn
DuPont Pharmaceuticals Company
Wilmington, DE, USA

18S Ribosomal RNA Detection on Northern Blot Employing a Specific Oligonucleotide

BioTechniques 31:1250-1252 (December 2001)

This manuscript describes a quick and simple method that facilitates 18S rRNA detection on a northern blot. It relies on the use of an oligonucleotide that specifically hybridizes with the 18S rRNA of a variety of species and shows no cross-hybridization with any mRNA. In contrast to current methods, the method described allows immediate reuse of the membranes for further gene expression studies after detection of the18S rRNA.

Northern blot analysis is a standard method to define and verify differential gene expression in various fields of research. To confirm equal loading, successful transfer of the RNA onto membranes, and for the normalization of signals of particular mRNAs, northern blots have to be rehybridized with a probe specific for a housekeeping gene or the 18S or 28S rRNA. In contrast to housekeeping genes, such as GAPDH or β-actin, the expression of which is often affected by experimental treatment or stage of development (1,2–6,8), cells typically maintain a constant level of rRNA, making rRNA an appropriate endogenous internal control (1,4,8). However, the disadvantage of rRNAs is their high G/C content resulting in a high melting temperature of the cDNA/RNA hybrid. Northern blots commonly can be rehybridized several times, but the cDNA for an rRNA should always be used as the last probe, since it can hardly be stripped from membranes. Therefore, the expression profile of the target gene is not available until the last hybridization is done.

I have designed an oligonucleotide appropriate for northern blot hybridization that can easily be stripped from membranes and specifically hybridizes with the 18S rRNAs of a variety of different species. The 20-mer with the sequence 5’-CGGAACGACGGTA TCTG-3’ has a relatively low G/C content and shows no cross-hybridization with any mRNA. The present study shows the results of a northern blot that was performed on total RNA isolated from tissue samples of human, rat, mouse, and rabbit origin, according to the procedure of Chomczynski and Sacchi (3). Fifteen micrograms of total RNA were size-fractionated per slot on a 1% agarose gel containing 0.66 M formaldehyde. After capillary transfer to a Hybond-N+ membrane (Amer sham Pharmacia Biotech, Piscataway, NJ, USA) using 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) as the transfer buffer, the RNA was fixed to the filter by means of a model 1800 UV Stratalinker™ (Stratagene, La Jolla, CA, USA). For northern blot analysis, membranes were prehybridized for 30 min at 42°C using an ExpressHyb™