SHORT TECHNICAL REPORTS

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Efficient DNA Subcloning through Selective Restriction Endonuclease Digestion

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ABSTRACT

Described here is a selective restriction endonuclease digestion method that eliminates the electrophoresis step that is usually used during the subcloning of new DNA sequences into typical E. coli*-based plasmids. The method increases yield while decreasing laboratory resource and time utilization. By using donor and acceptor sequences that contain unique restriction sites found only outside of the intended recombination sequences, the initial digestion products can be directly combined without electrophoresis if the ligation step is followed by a selective digestion using the unique restriction enzymes before transformation. This system is based on the several order of magnitude decrease in transformation efficiency of linearized compared to circular plasmids. As an example, this method was used to obtain recombinants between a 3.6 kb acceptor plasmid and 3.0 kb insert following one ligation reaction after the failure of nine standard reactions using similar amounts of input DNA. It is particularly applicable to situations in which low subcloning efficiencies are expected. The technique can be extended to a large percentage of planned recombinations by using nonidentical compatible cohesive or blunt-ended fragments, or site-directed mutagenesis.*

INTRODUCTION

The subcloning of new DNA sequences into bacterial plasmids using restriction endonuclease-based recombination forms the backbone of modern molecular biology. The standard or most commonly used method for subcloning is *(i)* to digest the insert and acceptor plasmid source DNA sequences with complementary restriction enzymes; *(ii)* separate the intended sequences from the unwanted background sequences using agarose gel electrophoresis; *(iii)* stain and excise slices carrying the plasmid bands; *(iv)* recover the selected DNA from the gel using electroelution or spin-column methods; *(v)* combine and ligate the sequences and then *(vi)* transform competent bacterial cells with the product, followed by the recovery and assay of colonies (2). One of the most time- and resource-intensive steps of this process is gel electrophoresis and recovery of the intended component sequences. This step also results in loss of input DNA, which decreases the number of output colonies and the probability of successful subcloning (2).

Circularized plasmid DNA transforms competent *E. coli* at approximately 1000-fold the efficiency of the same plasmid when it has been linearized by a single restriction endonuclease cut (1). Thus, if competent *E. coli* are transformed with a DNA mixture containing an intended circularized plasmid and an unwanted linearized plasmid, there will be a marked preference in the output colonies for the intended plasmid. Any other nonplasmid species (i.e., those that do not contain the *E. coli* origin of replication and antibiotic resistance gene) will not produce viable colonies on the standard antibiotic-agar plate. To increase the yield of low-efficiency recombinations and also decrease the time and resources needed to accomplish a subcloning procedure, we used these characteristics to search each set of donor and acceptor plasmids for restriction sites unique to the unwanted background sequences (donor plasmid backbone carrying the insert and excised sequence from acceptor plasmid). Then we eliminated the gel electrophoresis step and combined the initial digestion mixtures directly; this was followed by ligation and a selective digestion of the unintended recombination products and the unique restriction enzymes before transformation.

Heat-inactivation or phenol extraction was used at each step to eliminate contradictory restriction endonuclease or ligase activity during subsequent steps. The results were so encouraging that we created additional techniques to make the procedure more universally applicable and designed plasmids with sites specifically for this purpose. This method has been used in the construction of a number of plasmids in the laboratory, one of which is presented here.

MATERIALS AND METHODS

pCON

Standard recombination techniques were used in an attempt to insert 3.0 kb of sequence containing the gene for gC from the 5.6 kb pUC19 based donor plasmid pgC43 into the 3.6 kb pBluescript II® (Stratagene, La Jolla, CA, USA) based HSV-1 amplicon acceptor plasmid pBON, to create the 6.5 kb plasmid pCON (Figure 1). pgC43 and pBON (7.5 µg each) were first digested with *Pst*I and *Hin*dIII (New England Biolabs, Beverly, MA USA) in a 50 µL reaction mixture following the manufacturer's protocol. Next, 20 µL of the digestion mixtures were loaded into 1% agarose gels and the bands were electrophoretically separated. Agarose strips containing the gC and pBON backbone sequence bands were located through ethidium bromide staining of a side strip of the gel and excised. The gel strips were then placed in a dialysis bag with TAE buffer (Tris-acetate/EDTA) and electroeluted at 5 V/cm for 2 h. DNA was then phenol/chloroform extracted from the TAE solution, precipitated with isopropanol/sodium acetate/glycogen, washed in ethanol and dissolved in 10 µL distilled water.

As an alternate extraction method, the gel strips were diced, frozen at -20°C for 1 h, transferred to a RPM™ miniprep spin filter (BIO 101, Vista, CA, USA) with 100 µL TE, spun at 13 000× *g* for 5 min in a microcentrifuge (Eppendorf, Hamburg, Germany), ethanol precipitat-

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Protocol

1. Digest donor and acceptor DNA with restriction enzymes for the intended recombination sites in appropriate buffers. [a]The selective restriction enzymes for the sites in the unwanted sequences may also be included here.
2. [b]Heat inactivate enzymes in digestion mixtures at 65°C for 20 min (adjust the temperature and time appropriately for the individual enzymes).
3. Combine the digestion mixtures in appropriate concentrations with T4 DNA ligase and appropriate buffers.
4. Heat-inactivate the ligation mixture at 65°C for 10 min.
5. Dilute the ligation mixture into the appropriate selective restriction enzyme buffers (1:5); add selective restriction enzymes for the sites in the unwanted sequences, and digest[a].
6. [a,b]Heat-inactivate the digestion mixture.
7. Transform the competent *E. coli* with your method of choice.

[a]Optional step: adjust time as appropriate for complete digestion by selected enzymes (10 min to overnight)

[b]Phenol extraction may be used to remove restriction enzymes that are not heat-inactivated.

ed, and the pellet dissolved in 10 μL distilled water. (The QIAquick™ kit from Qiagen, Hilden, Germany has since been used instead.) Increasing ratios of acceptor and insert DNA solutions from 1:1 to 1:6, with combined solution volumes from 2–18 μL, were added to a T4 DNA ligation mixture to a total volume of 20–21 μL per the manufacturer's protocol, and incubated overnight at 15°C. DH5α competent cells were transformed with 3 μL ligation mixture per 200 μL cells using the heat-shock method, and plated on ampicillin-agar plates. Colonies were selected and grown in LB media with ampicillin and the DNA was isolated using the RPM spin filter. To analyze the resultant DNA, it was electrophoresed on 1% agarose gel with ethidium bromide, first undigested then digested with *Pst*I and *Hin*dIII, *Rsr*I and/or *Nru*I (New England Biolabs) if of appropriate size.

The selective restriction method was subsequently used to perform the same recombination. *Pst*I and *Hin*dIII were removed by phenol extraction from 10 µL of the digestion of pgC43 and pBON produced above. The resulting samples were added to the T4 DNA ligation mixture to a total volume of 20 µL per the manufacturer's protocol and incubated overnight at 15°C. The ligase was heat-inactivated by incubation at 65°C for 10 min. The unwanted pUC19 plasmid backbone of pgC43 contains a unique *Aat*II site, and the unwanted *Pst*I-*Hin*dIII fragment from pBON contains a unique *Spe*I site (Figure 1).

Four microliters of the ligation mixture were diluted into a 20 µL digestion mixture of *Aat*II and *Spe*I with appropriate buffers following the manufacturer's protocol and digested at 37°C. The selective digestion mixture was heat-inactivated by incubation at 65ºC for 20 min. DH5α competent cells were transformed with 3 µL of the product per 200 µL cells using the heat-shock method, and plated on ampicillin-agar plates. Colonies were selected and grown in LB media with ampicillin and the DNA subsequently isolated using the RPM spin filter. To analyze the resultant DNA, it was electrophoresed on 1% agarose gel with ethidium bromide, first undigested then digested with *Pst*I and *Hin*dIII, *Rsr*I and/or *Nru*I if successful ligation occurred.

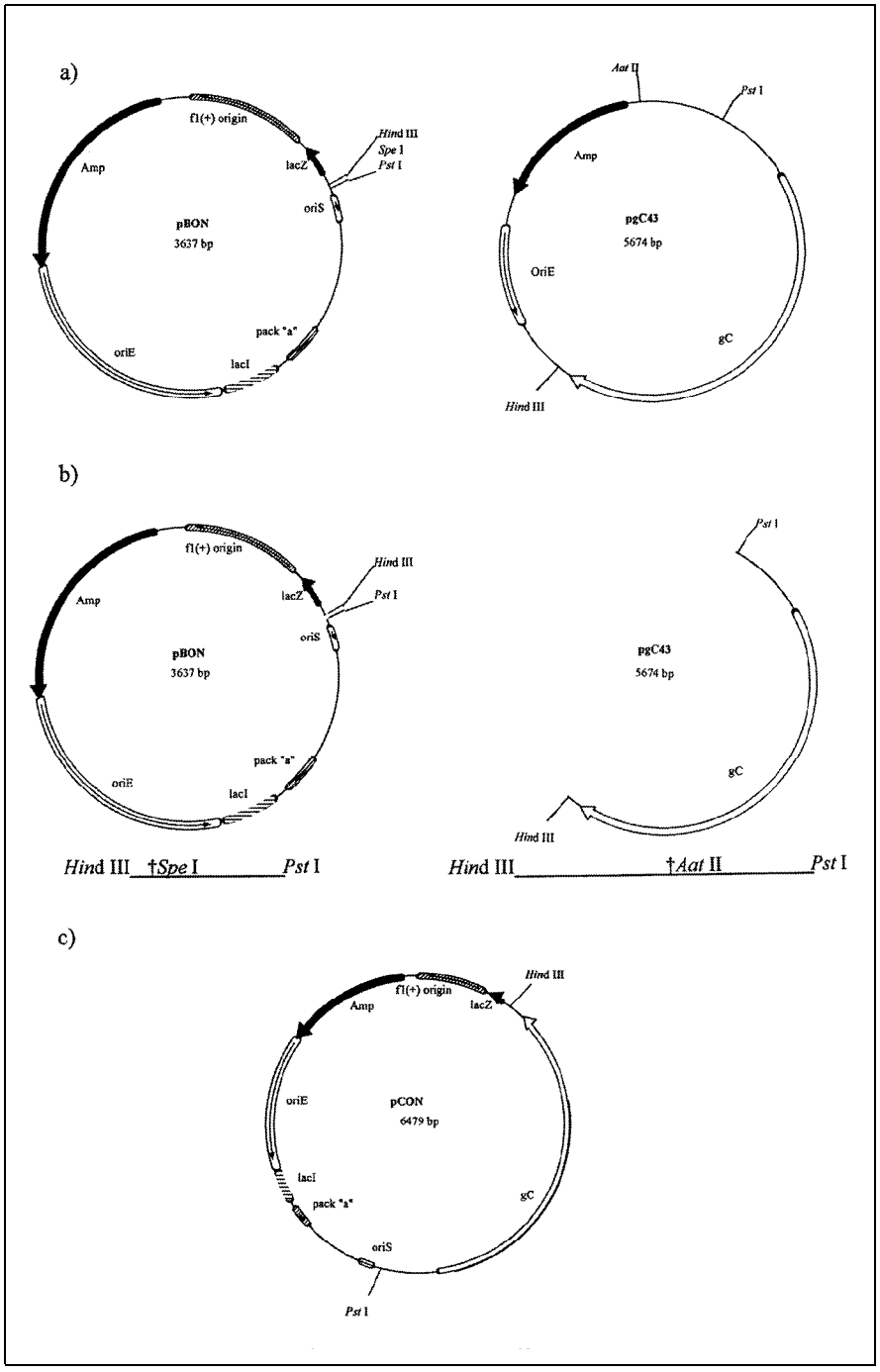

Figure 1. Subcloning of gC from pgC43 donor plasmid into pBON acceptor plasmid to create pCON product plasmid using the selective restriction method. (a) The 3.6 kb pBluescript II-based HSV-1 amplicon acceptor plasmid pBON carries the standard *E. coli*-based plasmid constituents, the HSV-1 origin of replication (oriS) and packaging sequence (pack), and a multiple cloning site containing *Pst*I and *Hin*dIII sites. The unwanted *Pst*I-*Hin*dIII fragment from pBON contains a unique *Spe*I site. The 5.6 kb pUC19-based pgC43 carries 3.0 kb of sequence between *Pst*I-*Hin*dIII containing the gene for the HSV-1 heparan sulfate binding protein gC. The unwanted pUC19 plasmid backbone of pgC43 contains a unique *Aat*II site. (b) Products after digestion with *Pst*I and *Hin*dIII. Products are mixed and ligated. Selective restriction digestion is then conducted with *Aat*II and *Spe*I to make single cuts in recombination products carrying the unwanted sequences. (c) Remaining pCON product carrying the gene for the HSV-1 heparan sulfate attachment protein gC from pgC43 in the pBON HSV-1 plasmid backbone.

RESULTS AND DISCUSSION

Using standard techniques to insert gC into pBON to create pCON, nine ligation and transformation reactions were attempted and plated on 27 ampicillin-agar plates. Electroelution was used to extract DNA for seven of the ligations and spin filters were used for two of the ligations. Electrophoresis and DNA recovery from gels required approximately 4–5 h for electroelution and 3 h for spin filters. From the nine transformations and 27 plates, a total of five colonies was produced. With analytic electrophoresis, DNA preparation from three of these colonies produced weak staining bands of inappropriate size, and two produced bands of ap-

proximately the correct size, but having an incorrect digestion pattern for pCON with *Pst*I and *Hin*dIII, *Rsr*I or *Nru*I.

The selective restriction method that was subsequently used to create pCON produced 19 colonies on the first ampicillin-agar plate. With electrophoresis, DNA preparation from two of these colonies produced strong DNA bands of the appropriate size for pCON, eight produced bands consistent with unaltered pBON and nine produced insufficient amounts of product. Both DNA preparations consistent with pCON produced appropriate restriction patterns with *Pst*I, *Hin*dIII, *Rsr*I and *Nru*I. One was arbitrarily chosen for further characterization and use.

Insertion of gC was confirmed with fluorescent dye-tag sequencing. This pCON sample was then used to create a plasmid carrying enhanced green fluorescent protein (EGFP) and Neo^r (pCONG). pCONG has been demonstrated to produce a functional HSV-1 vector as before through transfection of mammalian cells with EGFP. Production of gC from pCONG has been confirmed with Western analysis. Excluding ligation, the selective restriction method required approximately 2 h, most of which was incubation time. The Genetics Computer Group mapplot program (University of Wisconsin) was used to generate site-sorted linear plasmid restriction maps for both subcloning strategies. Approximately 10 min were required to choose the initial enzymes (*Pst*I and *Hin*dIII) for examination.

This method has subsequently been used to successfully conduct a number of recombinations using various plasmids. Of specific technical interest, site-directed mutagenesis was used to insert a unique *Asc*I site into pCONG, thus creating pCONGA with unique *Asc*I and *Eco*NI sites flanking the gC heparan sulfate binding domain. Oligonucleotides coding for a His tag and unique *Hpa*I site were recombined into the *Asc*I and *Eco*NI sites to create pCONGAH. The *Hpa*I site was included to allow the use of the selective restriction method to quickly replace the *Asc*I-*Eco*NI fragment with targeting epitopes, as well as to allow restriction digestion confirmation of product plasmids. To construct CONGA4, a 92 bp oligonucleotide coding for the receptor binding domain of IL-4 and having termini complementary to the *Asc*I and *Eco*NI sites of pCONGAH was synthesized (Life Technologies, Rockville, MD, USA) and recombined into pCONGAH using an *Hpa*I selective digestion after the failure of standard methods.

These results indicate that the selective restriction method may produce a larger yield of successful recombinations than the most commonly used methods, while using less research time and supplies. The differences in yield

seen here might not be extrapolated to other subcloning protocols, as they have been compared only with the pCON-based plasmids. Since this method has produced favorable outcomes in the production of other plasmids, however, it appears to have a relatively broad usefulness. It may be most useful for difficult recombinations such as those using large plasmids. Of interest, the recombination yields described here and using the standard method appeared lower than those using other plasmids. This may be specific to the input sequences, especially those originating from HSV-1, which contain high GC content and homologous regions. As the probability of a mixture of plasmid fragments being successfully ligated into a functional circular plasmid is further inversely dependent on the number of fragments, including the selective restriction enzymes in the initial digest mixture may increase the relative yield of intended recombinants.

This method cannot necessarily be applied to all recombinations because it presumes the existence of unique restriction sites in the unwanted sequence of both the donor plasmid of the insert and the excised portion of the acceptor plasmid. However, this requirement is frequently fulfilled, particularly when enzymes having multiple sites in the unwanted sequence are considered. Another option is to use restriction enzyme-producing fragments, that when ligated, result in a novel sequence that is not cleaved by the initial enzymes. Finally, if frequent repetition of similar recombinations using one or both of the same sources is expected, the sources can be designed to include unique sites using techniques such as site-directed mutagenesis.

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Matthew A. Spear
University of California at San Diego
San Diego, CA, USA

DNA Extraction Method for Screening Yeast Clones by PCR

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ABSTRACT

A simple procedure for isolating yeast DNA suitable for use as a template for PCR amplification is described. SDS treatment alone is sufficient for extraction of chromosomal DNA from yeast cells. Cells of a yeast colony are suspended in a small volume (about 20 mL) of a 0.25% SDS solution, mixed vigorously and centrifuged. The supernatant can be directly used as a template after dilution to give an SDS concentration of less than 0.01% in the final PCR mixture.

INTRODUCTION

Screening of yeast clones for a sequence of interest is a routine step in yeast DNA manipulations. To obtain insertions in a gene of interest, it is necessary to screen several candidates to identify one with the desired modification. This screen can be performed using Southern blot hybridization, but the method is time consuming and requires expensive reagents. In contrast, PCR is a simple, fast and inexpensive method for this purpose. The use of yeast DNA as a PCR template can be prepared by several methods involving either enzymatic digestion of the cell wall (8) or mechanical disruption by glass beads (1,3). For screening many transformants by PCR, simple DNA isolation procedures have been reported such as the direct use of yeast cells (9,10), alkali extraction (12), glass bead disruption (6) and enzymatic digestion (2,7). In our experience, however, some of these methods were not reproducible (13) and others were not efficient for screening large numbers of yeast clones. Here, we describe a rapid and reliable procedure for isolating yeast DNA that can be used directly for PCR.