Highly efficient yeast-based in vivo DNA cloning of multiple DNA fragments and the simultaneous construction of yeast/*Escherichia coli* shuttle vectors

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In vivo recombinational cloning in yeast is a very efficient method. Until now, this method has been limited to experiments with yeast vectors because most animal, insect, and bacterial vectors lack yeast replication origins. We developed a new system to apply yeast-based in vivo cloning to vectors lacking yeast replication origins. Many cloning vectors are derived from the plasmid pBR322 and have a similar backbone that contains the ampicillin resistance gene and pBR322-derived replication origin for Escherichia coli. We constructed a helper plasmid pSU0 that allows the in vivo conversion of a pBR322-derived vector to a yeast/E. coli shuttle vector through the use of this backbone sequence. The DNA fragment to be cloned is PCR-amplified with the addition of 40 bp of homology to a pBR322-derived vector. Cotransformation of linearized pSU0, the pBR322-derived vector, and a PCR-amplified DNA fragment, results in the conversion of the pBR322-derived vector into a yeast/E. coli shuttle vector carrying the DNA fragment of interest. Furthermore, this method is applicable to multifragment cloning, which is useful for the creation of fusion genes. Our method provides an alternative to traditional cloning methods.

INTRODUCTION

Escherichia coli-based traditional cloning methods use restriction enzyme cleavage followed by DNA joining with DNA ligase (1). These methods are often inefficient and laborious, particularly for the cloning of multiple DNA fragments or a large DNA fragment. On the other hand, yeast cells are an ideal tool for conducting efficient in vivo recombinational cloning (2). Because most animal, insect, and bacterial vectors carry no yeast replication origins, researchers have previously been limited to using yeast-based in vivo cloning procedures for the construction of vectors in experiments involving yeast; for example, yeast two-hybrids (3,4). In order to clone a desired DNA fragment into the vectors lacking yeast replication origins, the yeast-based in vivo cloning method usually requires two rounds of DNA manipulation. In the first round, the vector is converted to a yeast/*E. coli*

shuttle vector by integrating the replication origin and a selectable marker for yeast using an *E. coli*-based traditional cloning method. In the second round, the desired DNA fragment is PCR-amplified with 20–40 bp of homology to a yeast vector (2,5) and is cotransformed with the linearized vector into yeast.

To eliminate these two rounds of lengthy DNA manipulation, we developed a new yeast-based in vivo cloning method. Many cloning vectors are derived from the pBR322 plasmid and contain a common backbone sequence carrying an ampicillin resistance (Amp^r) gene and a pBR322-derived replication origin for *E. coli* (pBR322 origin) (6). We constructed a helper plasmid pSU0 that allows the in vivo conversion of a pBR322-derived vector to a yeast/*E. coli* shuttle vector through the use of this backbone sequence. Cotransformation of pSU0 into yeast with a pBR322-derived vector and the desired DNA fragment

results in the conversion of the pBR322-derived vector into a yeast/*E. coli* shuttle vector and simultaneously allows the cloning of the DNA fragment into the same vector. In addition, we show that this method is applicable to multifragment cloning (7). Our new method can replace lengthy traditional cloning methods with an efficient one-step protocol.

MATERIALS AND METHODS

Strains

E. coli strain DH5 α was used for the propagation of plasmids, and the strain BL21 StarTM (DE3) (Invitrogen, Carlsbad, CA, USA) was used for the expression of recombinant proteins. Yeast strain YPH499 (*MATa ura3-52 lys2-801^{amber} ade2-101^{orchre} trp1- Δ 63 his3- Δ 200 leu2- Δ 1*) was used for homologous recombination.

Construction of pSU0

The helper plasmid pSU0 (GenBank[®] accession no. AB215109; Figure 1A) was constructed by yeast homologous recombination. This plasmid has an Amp^r gene, a 2 μ yeast replication origin (2 μ origin), a *URA3* selectable marker, and a pUC-derived replication origin for *E. coli* (pUC origin) (Figure 1A). There is a single nucleotide difference between the pBR322 and pUC origins (8). First, to rearrange these origins and genes in the desired order, they were PCR-amplified from pYES2/CT (Invitrogen). For the PCR amplification of the Amp^r gene, 2 μ origin-*URA3*, and the pUC origin, the primer pairs used were pSU0-1 and pSU0-4 (15 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min using *Pfu* DNA polymerase; Fermentas, Vilnius, Lithuania), pSU0-2 and pSU0-5 (40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 3 min using *Pfu* DNA polymerase), and pSU0-3 and pSU0-6 (15 cycles of 95°C for 30 s, 55°C for 30s, and 72°C for 1 min using *Pfu* DNA polymerase), respectively (Table 1). The primer pSU0-1 was designed to provide *EcoRI* and *BamHI* sites. Next, in order to provide the long overlapping sequence

to these fragments, two adjacent fragments were joined by the PCR-based overlap extension method (9) as follows. Using PCR, the *Amp^r* and 2 μ origin of replication-*URA3* selectable marker fragments, 2 μ origin-*URA3* and the pUC origin fragments, and the pUC origin and *Amp^r* fragments were joined by the pSU0-1 and pSU0-5, pSU0-2 and pSU0-6, and pSU0-3 and pSU0-4 primer pairs, respectively (45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C

for 4 min using *Pfu* DNA polymerase). Cotransformation of these three DNA fragments into yeast (10) resulted in homologous recombination at the overlapping sequences and the consequent formation of the plasmid pSU0. The proper formation of pSU0 was confirmed by restriction analysis (data not shown). For the cloning experiments, the plasmid pSU0 was digested with *Bam*HI and *Eco*RI and purified by electrophoresis.

PCR Amplification of GFP and RFP Genes

Open reading frames (ORFs) of green fluorescent protein (GFP) and red fluorescent protein (RFP) genes were obtained by PCR using the plasmids pBAD-GFPuv (Clontech Laboratories, Mountain View, CA, USA; accession no. U62637) and pDsRed2 (Clontech Laboratories) as DNA templates, respectively. First, for the GFP cloning experiment, the GFP gene was PCR-amplified with primers pGFPF and pGFP2 (15 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min using Pyrobest[®] DNA polymerase; TAKARA BIO, Ohtsu, Shiga, Japan). Subsequently, to provide this GFP gene with ends homologous to pET32a(+) (Novagen, Madison, WI, USA) (Figure 1C), the GFP fragment was PCR-amplified with primers pSU0HindIII and pSU0XhoI, the 5' ends of which include 40-nucleotide sequences homologous to the multiple cloning site (MCS) of pET32a(+) (35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min using Pyrobest DNA polymerase). The pET32a(+) vector is designed for expression of the target protein fused with thioredoxin under the control of a T7 promoter (11). An *E. coli* strain, such as BL21 Star (DE3), carries a chromosomal copy of the T7 RNA polymerase gene under the control of the *lacUV5* promoter. The addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to this strain induces the expression of T7 RNA polymerase followed by the expression of the thioredoxin-fused target protein.

For the multifragment cloning experiment, the fragments of the GFP and RFP genes were designed to share overlapping 40-bp sequences at their junction and to include 40-bp sequences at their nonoverlapping ends that are homologous to the MCS of pET32a(+) (Figure 1D). To obtain these fragments, the GFP gene was PCR-amplified with the primers pGFPF and pGFP2 (15 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min using Pyrobest DNA polymerase) and subsequently with the primers pSU0HindIII and pSU0XhoI (35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min using Pyrobest DNA polymerase). RFP was

Figure 1. Diagram of the yeast-based in vivo cloning system. (A) Map of the helper plasmid pSU0. (B) The diagram of in vivo conversion of a pBR322-derived vector to a yeast/*Escherichia coli* shuttle vector. (C and D) The diagrams of simultaneous conversion of the pET32a(+) to a yeast/*E. coli* shuttle vector and cloning of (C) a GFP fragment or both (D) GFP and RFP fragments into the vector. GFP, green fluorescent protein; RFP, red fluorescent protein; MCS, multiple cloning site.

Table 1. Primers Used in this Study

Name	Sequence
pSU0-1	5'-CGGGATCCATCGGAATTCATATTGAAAAAGGAAGAGTATG-3'
pSU0-2	5'-TGAAGATCCTTTTTGATAATAATCTTCCTGTTTTGGGGC-3'
pSU0-3	5'-AATTATATCAGTTATTACCCCTCATGACCAAAATCCCTTA-3'
pSU0-4	5'-GCCCCAAAAACAGGAAGATTATTATCAAAAAGGATCTTCA-3'
pSU0-5	5'-TAAGGGATTTTGGTCATGAGGGGTAATACTGATATAATT-3'
pSU0-6	5'-ATGAATTCGATGGATCCCGGCGGTAATACGGTTATCCAC-3'
pGFPF	5'-TTCGAGCTCCGTCGAGCTAGCAAAGGAGAAGAACTTTTCA-3'
pGFPR	5'-ATGACGTTCTCGGAGGAGGCTTTGTAGAGCTCATCCATGC-3'
pGFPR2	5'-AGCAGCCGGATCTCATTATTGTAGAGCTCATCCATGCCA-3'
pRFPF	5'-GCATGGATGAGCTCTACAAAGCCTCCTCCGAGAACGTCAT-3'
pRFPR	5'-AGCAGCCGGATCTCACTACAGGAACAGGTGGTGGCGGCC-3'
pSU0HindIII	5'-GGCCATGGCTGATATCGGATCCGAATTCGAGCTCCGTCGA-3'
pSU0XhoI	5'-ACTCAGTTCCTTTTCGGGCTTTGTTAGCAGCCGGATCTCA-3'

also amplified with primers pRFPF and pRFPR (15 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min using Pyrobest DNA polymerase) and subsequently with the primers pRFPF and pSU0XhoI (35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min using Pyrobest DNA polymerase). These PCR-amplified DNA fragments were electrophoretically purified and used for cloning into pET32a(+).

Yeast and *Escherichia coli* Transformation

For efficient cloning (12), the pET32a(+) vector was digested with *HindIII* and *XhoI* in its MCS and with *PstI* in its Amp^r gene. The digested vector was not purified further. After overnight incubation of yeast cells in 5 mL of double-strength yeast extract-peptone-adenine-dextrose (2× YPAD) broth at 30°C, they were inoculated into 50 mL of 2× YPAD broth and grown to obtain a cell density of 1.7×10^7 cells/mL. The yeast cells were harvested and transformed by the method of Gietz and Woods (10). However, the transformation procedure was a quarter scale of the published method. These cells were cotransformed with the linearized pSU0, digested pET32a(+), and the relevant PCR fragments. Fifty nanograms of the digested pET32a(+) correspond to 1 in the molar ratio of mixed DNA shown in Table 2. The transformants were selected using synthetic complete plates lacking

uracil, incubated at 30°C. We obtained more than 1000 transformants per 50 ng of the digested pET32a(+) in the GFP cloning experiment. We also obtained about 300 transformants per 50 ng of the digested pET32a(+) in the multifragment cloning experiment. All colonies were scraped from the plates with plating beads, and the collected yeast cells were disrupted by acid-washed glass beads (425–600 µm; Sigma-Aldrich, Steinheim, Germany). The plasmids were purified from the disrupted cells using a Mini-M[®] Plasmid DNA Extraction System (Viogene, Taipei, Taiwan). The rescued plasmids were transformed into *E. coli* DH5α for propagation. The propagated plasmids were then transformed into *E. coli* BL21 Star (DE3), and plated on agar containing 100 µg/mL ampicillin and 0.01 mM IPTG. The colonies expressing fused protein were counted.

RESULTS AND DISCUSSION

Cotransformation of the linearized helper plasmid pSU0 (Figure 1A) into yeast with the pBR322-derived vector that has been digested in the backbone sequence results in homologous recombination (Figure 1B). Using pSU0, we developed a new yeast-based in vivo cloning method that allows simultaneous conversion of a pBR322-derived vector into a yeast/*E. coli* shuttle vector and cloning of DNA inserts of interest

by one-step transformation of yeast (Figure 1C).

To test this system, we cloned the GFP gene into an *E. coli* expression vector, pET32a(+). First, PCR was used to amplify the ORF of the GFP gene with 40 bp of homology to the MCS of pET32a(+), such that homologous recombination would create an in-frame fusion of GFP with thioredoxin. Subsequently, to clone the GFP into pET32a(+), yeast was cotransformed (10) with the GFP gene, the digested pET32a(+), and the linearized pSU0 (Figure 1C). In this experiment, these DNA fragments were mixed at various molar ratios (Table 2). We rescued the plasmids from all yeast transformants. The rescued plasmids were transformed into *E. coli* DH5α for propagation because the protein expression host, BL21 Star (DE3), does not have high transformation efficiency. The propagated plasmids were then transformed into the BL21 Star (DE3), and colonies expressing thioredoxin-fused GFP were counted (Table 2). The difference in the ratio of DNA fragments added had little effect on the recombination frequency. Under any conditions, more than 80% of all transformants expressed thioredoxin-fused GFP. To compare this new method with the conventional yeast-based in vivo cloning method, yeast was also cotransformed with a GFP fragment and pET32a(+) carrying the yeast 2µ origin of replication and yeast selectable marker, *URA3*, which were mixed in a 1:1 molar ratio. Subsequent analysis of the rescued plasmids showed that 91% have the ability to express thioredoxin-fused GFP. Thus, little difference was detected in recombination efficiency between the two methods.

Restriction analysis was used to examine four colonies expressing thioredoxin-fused GFP, which were obtained by the new method. The plasmids were extracted, digested with *EcoRI* and *DraIII*, and analyzed by electrophoresis. In all lanes, 4.6, 3.6, and 1.2 kb fragments were present (Figure 2A, lanes 1–4). This pattern matched the predicted pattern (Figure 2B). DNA sequencing of these clones also confirmed the correct integration of the GFP gene (data not shown).

Table 2. The Efficiencies of One-Step Yeast-Based In Vivo Cloning

Molar Ratio of Mixed DNA pET32a(+):pSU0:GFP	Percentage of <i>Escherichia coli</i> Colonies Expressing GFP
1:1:1	88
1:1:6	88
1:1:12	90
1:6:1	85
1:12:1	88
6:1:1	84
12:1:1	83

Each experiment was repeated three times, and the averages are shown.

To determine how clones not expressing thioredoxin-fused GFP were generated, we examined plasmids from four such clones. Interestingly, some of them showed the same restriction pattern as those from colonies expressing thioredoxin-fused GFP (Figure 2A, lanes 5, 7, and 8). DNA sequencing indicated that three clones showed frameshift mutations at the sites targeted by the PCR primers. The fourth clone not expressing thioredoxin-fused GFP was created by the incorrect joining of pSU0 and the *XhoI-PstI* fragment of pET32a(+) by homologous recombination and nonhomologous end-joining (Figure 2A, lane 6) (13).

One of the most important advantages of yeast-based in vivo cloning is that it allows one to clone multiple PCR-amplified fragments with high efficiency (7). In order to evaluate this new method, we cloned the GFP and RFP genes simultaneously into pET32a(+) (Figure 1D). We amplified the ORFs of GFP and RFP and added 40 bp overlaps by PCR, such that homologous recombination would create in-frame fusion of thioredoxin, GFP, and RFP (Figure 1D). Because varying ratios of the DNA fragments had little effect on the recombination frequency

in the GFP cloning experiment, yeast was transformed (10) with the GFP fragment, RFP fragment, the digested pET32a(+), and linearized pSU0 mixed in a 1:1:1:1 molar ratio. The rescued plasmid was amplified in DH5 α cells and then transformed into BL21 Star (DE3) for recombinant protein expression. The colonies expressing thioredoxin-fused GFP-RFP were counted and 65% were found to express the fusion protein.

We examined four colonies expressing thioredoxin-fused GFP-RFP by restriction analysis. In all lanes, 4.6, 3.6, and 1.9 kb fragments were present (Figure 2C, lanes 1–4). This pattern matched the predicted pattern (Figure 2D). DNA sequencing of these clones also confirmed the correct integration of both genes (data not shown).

To determine the events responsible for the production of clones not

expressing thioredoxin-fused GFP-RFP, we analyzed plasmids from four colonies not expressing GFP-RFP. In one clone, a frameshift mutation occurred at the site targeted by PCR primers (Figure 2C, lane 7). Other errors included the conversion of pET32a(+), which was not digested in the MCS, to a yeast/*E. coli* shuttle vector (Figure 2C, lane 5); cloning of an N-truncated GFP with intact RFP into the vector (Figure 2C, lane 6); and integration of unwanted pDsRed and pBAD-GFPuv sequences into pSU0 (Figure 2C, lane 8).

The main factor responsible for clones not expressing fused protein was the occurrence of frameshift mutations at the sites targeted by the PCR primers (Figure 2A, lanes 5, 7, and 8; Figure 2C, lane 7). It is likely that errors in the synthesis of the PCR primers caused these mutations.

The new cloning method described here is highly efficient. It is applicable

Figure 2. Restriction analysis of plasmids from transformants that were obtained in the (A and B) GFP cloning and (C and D) multifragment cloning experiments. (A and C) Plasmids from transformants were digested by *DraIII* and *EcoRI* and analyzed by electrophoresis on a 1% agarose gel. The GeneRuler™ 1 kb DNA Ladder (Fermentas) is indicated on the right (M). (B) Diagram showing the restriction patterns of the pET32a(+) containing the 2 μ origin of replication, *URA3* selectable marker, and GFP gene. (D) Diagram showing the restriction patterns of the pET32a(+) containing 2 μ origin of replication, *URA3* selectable marker, and both GFP and RFP genes. GFP, green fluorescent protein; RFP, red fluorescent protein.

to multifragment cloning, allowing fusion genes to be easily obtained by a one-step transformation of yeast. This provides a clear alternative to the traditional *E. coli*-based cloning methods using restriction enzyme cleavage followed by DNA joining with DNA ligase.

Many *E. coli*-based in vivo cloning methods have been previously reported (14,15). However, for efficient cloning in *E. coli*, special genotypes and electroporation apparatus are needed for transformation. In addition, efficient cloning in *E. coli*-based methods requires longer homologous sequences than those used in yeast-based methods (14,15). Unlike in *E. coli*, there is little need for special genotypes for in vivo cloning in yeast. Most yeast strains, including those used for two-hybrid methods, have a high rate of recombination, and yeast transformation does not require an electroporation apparatus for efficient transformation. Thus, our new cloning method is readily available to researchers conducting yeast two-hybrid experiments.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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