System of centromeric, episomal, and integrative vectors based on drug resistance markers for *Saccharomyces cerevisiae*

Christof Taxis and Michael Knop
EMBL, Heidelberg, Germany

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*Integrative, centromeric, and episomal plasmids are essential for easy, fast, and reliable genetic manipulation of yeast. We constructed a system of shuttle vectors based on the widely used plasmids of the pRS series. We used genes conferring resistance to Geneticin® (*kanMX4*), nourseothricin (*natNT2*), and hygromycin B (*hphNT1*) as markers. The centromeric and episomal plasmids that we constructed can be used the same way as the traditional auxotrophic marker-based shuttle vectors (pRS41x and pRS42x series). Additionally, we created a set of nine yeast integrative vectors with the three dominant markers. These plasmids allow for direct integration in the* LEU2, URA3, *and* HIS3 *locus of any yeast strain and the concomitant partial deletion of the gene. This prevents multiple integrations and allows for the rapid identification of correct integrants. The set of new vectors considerably enhances the flexibility of genetic manipulations and gene expression in yeast. Most notably, the new vectors allow one to work with natural yeast isolates, which do not contain auxotrophic markers.*

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a widely used experimental system for basic research in cell biology. Numerous researchers use it because of the ease of genetic manipulations. Two different techniques are often used for genetic manipulations. One is the use of vectors such as the integrative, centromeric, and episomal plasmids of the pRS series (1,2) for gene cloning and the second is the use of drug resistance markers for the deletion or tagging of genes (3–13). The latter technique has the advantage of being independent of the presence of auxotrophic markers in the yeast background. The common vectors used for yeast manipulation lack this advantage. They rely on the presence of an appropriate auxotrophic marker. This led to the construction of so-called designer deletion strains to combine several auxotrophic markers into one single yeast strain (1,14,15). Due to experimental conditions, however, it is not always possible to use one of these yeast strains, or the experiment may require growth on rich media in which plasmid selection is not possible. To circumvent these problems, we constructed a complete set of integrative, centromeric, and episomal plasmids based on the pRS series of plasmids containing the three different dominant drug resistance markers Geneticin® (*kanMX4*), nourseothricin (*natNT2*), and hygromycin B (*hphNT1*). The centromeric and episomal plasmids can be used similarly to conventional plasmids. YPD (yeast extract, peptone, dextrose) medium supplemented with the appropriate drug is used for plasmid selection, instead of synthetic medium lacking the selectable marker.

The pRS30x series of integrative plasmids has some drawbacks. To prevent multiple integrations, which could lead to overexpression of the inserted gene, we changed the integration mechanism. The new plasmids do not create a tandem repeat of the auxotrophic marker with the integrated DNA sequence in the middle (3). Instead, they replace part of the auxotrophic marker with the drug resistance marker (*kanMX4*, *natNT2*, or *hphNT1*) and the DNA sequence of choice.

The plasmids and sequence files can be obtained by contacting the authors (e-mails: taxis@embl.de or knop@embl.de) or by sending a letter with a self-addressed envelope to Dr. Michael Knop, EMBL Cell Biology and Biophysics Unit, Meyerhofstr. 1, D-69117 Heidelberg, Germany. Standard mail is preferred.

MATERIALS AND METHODS

Construction of Plasmids

Standard techniques were used for DNA manipulations (16). The integrative plasmids are based on pRS303, pRS305, or pRS306 (1). First, the multicloning sites (MCS) of these plasmids were destroyed, followed by insertion of the drug resistance marker in the *HIS3*, *LEU2*, or *URA3* gene. The centromeric and episomal plasmids were derived from plasmids pRS416 and pRS426, respectively (1,2). The *URA3* gene and the flanking promoter and terminator regions in these plasmids were replaced by the three different dominant selection markers. A detailed description of the plasmid constructions is provided as supplementary material, which is available online at www.BioTechniques.com.

Yeast Strains and Growth Conditions

Yeast strain ESM356 (17) was used for in-yeast ligation by homologous recombination. YPD and synthetic dropout media were prepared as previously described (18). The following drug concentrations were added to standard YPD plates or liquid YPD medium after autoclaving and cooling to 60°C: 200 mg/L Geneticin Selective Reagent (G418; Invitrogen, La Jolla, CA, USA) (4) were added to select for *kanMX4*; 300 mg/L hygromycin B (InvivoGen, Toulouse, France) were added to select for *hphNT1*; and 100 mg/L nourseothricin (ClonNAT; Werner BioAgents, Jena-Cospeda, Germany) were added to select for *natNT2*. A sterile filtered stock solution was used in the case of ClonNAT, whereas Geneticin and hygromycin B were used as provided by the manufacturer.

Table 1. Centromeric and Episomal Plasmids

Name	Selection Marker	Replication Origin	Control Digest
pRS41H	*hphNT1*	*ARS/CEN*	*Eco*RI: 1769; 3620
pRS41K	*kanMX4*	*ARS/CEN*	*Hin*dIII: 1249; 3929
pRS41N	*natNT2*	*ARS/CEN*	*Xma*I: 1605; 3409
pRS42H	*hphNT1*	2μ	*Eco*RI: 1769; 4448
pRS42K	*kanMX4*	2μ	*Hin*dIII: 1249; 4757
pRS42N	*natNT2*	2μ	*Xma*I: 1605; 4237

Control digests to verify the plasmids can be made using the restriction enzymes indicated. The sizes of the resulting DNA fragments are given in base pairs. *ARS*, autonomously replicating sequence; *CEN*, centromeric sequence.

The following media composition was used for the double selection of an auxotrophic marker plasmid together with a dominant drug resistance plasmid. The recipe was adapted from the composition reported in the supplementary information (SGA Analysis: Media) to Reference 19. The main change compared with standard synthetic complete (SC) medium was the replacement of ammonium sulfate with monosodium glutamate as a nitrogen source. The medium contains 20 g/L glucose, 1.7 g/L yeast nitrogen base without ammonium sulfate and amino acids (Difco™; BD, Franklin Lakes, NJ, USA), 1 g/L monosodium glutamic acid, and 2 g/L amino acid drop-out powder (18) lacking the appropriate auxotrophic marker. The appropriate dominant drug should be added after autoclaving of the medium.

Yeast Transformation

The following protocol for yeast transformation has been previously described (9). It is based on the lithium acetate method (20). Yeast cells were inoculated from a fresh pre-culture and grown to a density of 0.5–0.7 A_{600} at 30°C (approximately 10^7 cells/mL) in YPD medium. Yeast cells were harvested by centrifugation (500× *g* for 3 min), washed once with 0.1–0.5 volumes of sterile water, and once with 0.1–0.2 volumes of sterile SORB [100 mM lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA/NaOH, pH 8.0, 1 M sorbitol (special grade for molecular biology; Merck, Whitehouse Station, NJ, USA), adjusted with acetic acid to a pH 8.0]. The cells were resuspended in 360 μL of sterile SORB per 50 mL of cell culture and 40 μL of carrier DNA [10 mg/mL salmon sperm DNA (Invitrogen) denatured at 100°C for 10 min and cooled on ice] were added. The cells were divided into appropriate aliquots and placed at -80°C (no shock freezing). Usually, 10 μL of competent cells were used for the transformation of a centromeric or episomal plasmid and 50 μL of cells for the transformation of an integrative plasmid. Thawed competent cells were added to a sterile 1.5-mL tube containing the DNA (maximal 2 μL plasmid DNA/10 μL of cells). The suspension was mixed well before a 6-fold volume of sterile polyethylene glycol (PEG; 100 mM lithium acetate, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40% PEG 3350) was added. Cells were mixed again and incubated at room temperature for approximately 30 min. Dimethyl sulfoxide (DMSO) was added to a final concentration of 10%. The cells were placed in a water bath at 42°C for 5–20 min (15 min works best with most strains) and sedimented (2–3 min at 500× *g*). The cells were washed once with YPD and resuspended in 3 mL of YPD. They were then incubated on a shaker for 4–6 h at 30°C and spread on a plate with the appropriate dominant drug selection marker.

Selection of drug resistance yeast clones on plates often required replica

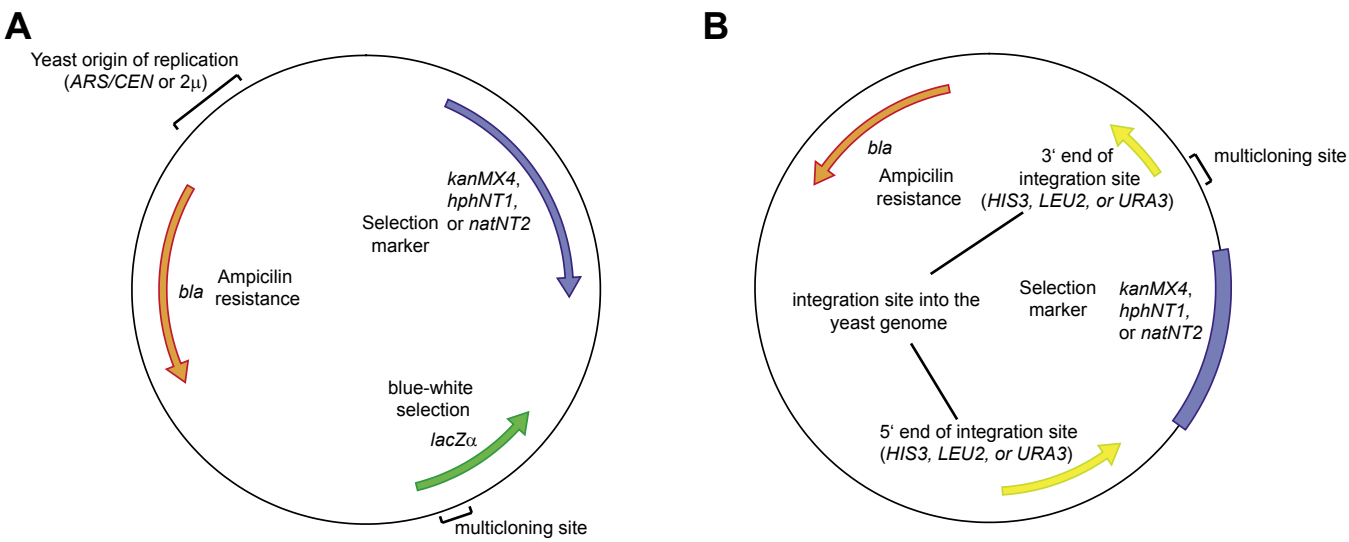

Figure 1. Features of the new episomal and centromeric plasmids (A) and the new integrative plasmids (B). The positions of the different selection markers and multicloning and integration sites are indicated. Detailed maps are provided as supplementary material (Figures S1 and S2) that is available online at www.BioTechniques.com. *ARS*, autonomously replicating sequence; *CEN*, centromeric sequence.

plating on the same selective medium after 2 days at 30°C due to the high background of transiently transformed cells (9,12).

RESULTS AND DISCUSSION

Centromeric and Episomal Plasmids with Dominant Selection Markers

Three different dominant drug resistance markers were used to replace the entire *URA3* sequence in the plasmids pRS416 and pRS426. The *kan, hph*, and *nat* markers confer resistance to Geneticin/G418 (6), hygromycin B, and nourseothricin (4), respectively. The three different resistance genes are under the control of the same promoter but differ in their terminators (5,6). Standard transformation procedures were used for the transformation into yeast. However, to allow expression of the drug resistance, a nonselective adaptation phase of 4–6 h was performed before spreading the transformed cells onto selective medium. We used the plasmids mainly for gene expression during sporulation. This differentiation process requires growth phases in liquid-rich medium to obtain synchronized sporulating cultures. We tested the plasmids with two different genes, *SSP1* and *SPO74*. We found that both genes, when harbored on a new centromeric plasmid, are able to complement the meiotic defects of the corresponding null-gene mutants. Using the episomal plasmids, we achieved overexpression of several genes during meiosis. We did not observe any differences in the new plasmids regarding transformation efficiency or plasmid loss when compared with the common pRS plasmids (unpublished observations). The plasmids have the same MCS as the other pRS plasmids (for restriction maps, see Supplementary Figure S1). This provides many opportunities to introduce DNA sequences. Because the backbone is the same as the other pRS plasmids, cloning of inserts from already existing pRS-based vectors into the new plasmids by homologous recombination is possible. An overview of the features of the new centromeric and episomal plasmids is shown in Figure 1A and in Table 1.

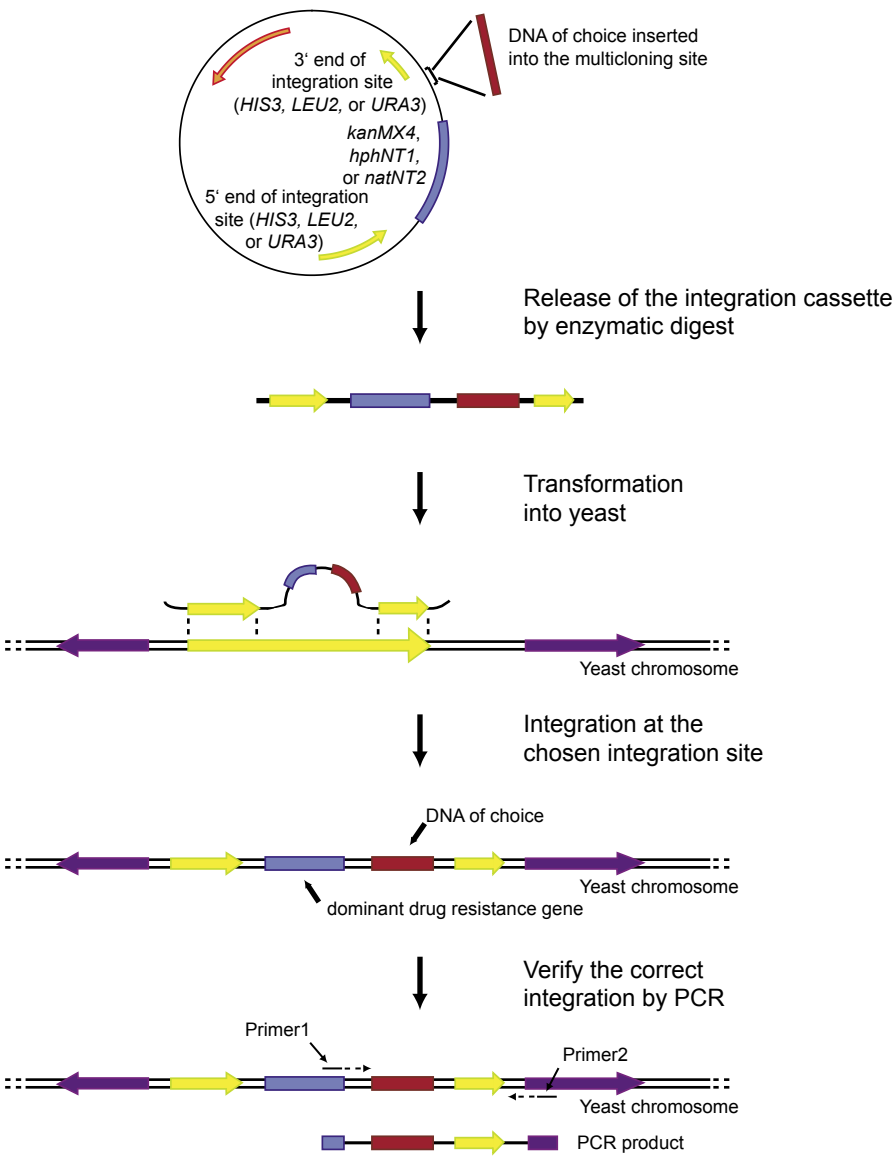

Figure 2. Step-by-step procedure to integrate DNA into the yeast genome using the newly created integrative plasmids. First, the DNA of choice has to be introduced into the multicloning site of one of the plasmids. This creates the cassette of drug resistance marker and DNA of choice flanked by regions homologous to *HIS3*, *LEU2*, or *URA3*. Then, the cassette is released from the vector by enzymatic digestion. Next, the linearized DNA is transformed into yeast. There it integrates at the selected auxotrophic marker locus. After selection for the drug resistance, the yeast clones can be tested by PCR for correct insertion.

The drug resistance marker-based centromeric or episomal plasmids confer an advantage by allowing the use of rich instead of synthetic media but are not limited to use in complete medium. Combination with common auxotrophic marker plasmids and growth in synthetic media is also possible. Another advantage of the new plasmids is their lack of dependency on a suitable auxotrophic marker in the target strain. This enables one to work with natural yeast isolates. Furthermore, if all the available auxotrophic markers in a yeast strain have been depleted due to genetic manipulations, the use of the dominant drug resistance plasmids is still possible. During preparation of this manuscript, we found that a similar approach was undertaken by Bardazzi and Casalone (21). In this work, the *URA3* gene of plasmid pRS416 was replaced by either *kanMX4* or *LEU4** (resistance to 5,5,5-trifluoro-DL-leucine). The plasmids created in our work are an addition to the available

Table 2. Integrative Plasmids

Name	Selection Marker	Integration Site	Control Digest
pRS303H	*hphNT1*	*HIS3*	*Hin*dIII+*Pvu*II: 356; 958; 4565
pRS303K	*kanMX4*	*HIS3*	*Hin*dIII+*Pvu*II: 356; 958; 1056; 3283
pRS303N	*natNT2*	*HIS3*	*Hin*dIII+*Pvu*II: 356; 958; 4182
pRS305H	*hphNT1*	*LEU2*	*Hin*dIII+*Pvu*II: 3067; 3613
pRS305K	*kanMX4*	*LEU2*	*Hin*dIII+*Pvu*II: 983; 2404; 3067
pRS305N	*natNT2*	*LEU2*	*Hin*dIII+*Pvu*II: 3067; 3230
pRS306H	*hphNT1*	*URA3*	*Hin*dIII+*Pvu*II: 356; 2659; 2765
pRS306K	*kanMX4*	*URA3*	*Hin*dIII+*Pvu*II: 356; 983; 1556; 2659
pRS306N	*natNT2*	*URA3*	*Hin*dIII+*Pvu*II: 356; 2382; 2659

Control digests to verify the plasmids can be made using the restriction enzymes indicated. The sizes of the resulting DNA fragments are given in base pairs.

vectors. Centromeric and episomal plasmids are now available with three different dominant drug resistance markers.

Yeast Integrative Plasmids with Dominant Selection Markers

We created a set of yeast vectors that can be used to integrate the DNA sequence of choice at three different genomic loci, namely the *URA3*, *LEU2*, and *HIS3*. The corresponding mutants are widely used as auxotrophic markers. Foreign DNA has been inserted at these loci since the creation of the first integrative yeast plasmids (YIp) (1,22). The integrative plasmids with the drug resistance markers are based on pRS303, pRS305, and pRS306 (1). The MCS of these plasmids were removed, and the drug resistance genes, together with a new MCS, were inserted in the auxotrophic marker genes. Restriction maps of the new integrative plasmids can be found in the supplementary material (Supplementary Figure S2). The restriction sites in the MCS are marked in blue. Additional cloning sites (in green) are included downstream of the selection marker. The integrative plasmids can be used for transformation as intact circular DNA if the appropriate restriction site is missing (unpublished observation), but using linearized DNA enhances the efficiency considerably. The plasmids were therefore cleaved before yeast transformation with suitable restriction enzymes to release a fragment containing the DNA insert and the selection marker flanked by sequences homologous to the respective target chromosomal locus. Upon transformation in yeast, this fragment is integrated into the genome at the auxotrophic marker locus as in a conventional gene deletion. A drawing of the mechanism is shown in Figure 2. This replacement mechanism represents the main difference between our integrative plasmids and the pRS30x plasmids, which form a tandem structure of the auxotrophic marker. The transformation can be carried out in the same way as the transformation of the centromeric and episomal vectors. Integration of two or three constructs at different auxotrophic marker locations is also possible. An overview of the features of the new integrative plasmids is shown in Figure 1B and in Table 2.

The commonly used integrative plasmids of the pRS series have two major disadvantages. First, transformation often leads to multiple integrations of the plasmid (3). There is no way to control this, and single integration events can only be faithfully validated using Southern blot analysis. Furthermore, the integration of a pRS30x plasmid generates a tandem structure of the auxotrophic marker. This can lead to recombination and subsequent loss of the plasmid. Moreover, selection for the integrated plasmid does not prevent recombination because the auxotropic marker can be repaired during the process. Under normal conditions, this recombination event is not very frequent. However, the integrated plasmid may contain DNA that provides a selective disadvantage to the cell. In this case, mixed populations may appear that contain cells with and without plasmid. All of these problems are eliminated with the use of the new set of plasmids because the mode of integration is changed to a conventional deletion of the auxotrophic marker. After the integration, the selection can be omitted without risking the loss of the inserted DNA sequence. Therefore, the new plasmids allow a more faithful integration of the desired DNA sequence into the yeast genome. A similar vector had been previously constructed (23), but insertion was restricted to the *HO* locus. The new integrative plasmids provide more possibilities and allow the integration of several different DNA sequences into the same yeast strain.

Another method of DNA integration has been developed recently, the so-called delitto perfetto (24,25). This method allows for the integration of DNA at any site into the yeast genome. This is mainly important for the generation of point mutations by transformation with oligonucleotides. Foreign DNA can be inserted at any point into the yeast genome by using PCR fragments with homologous flanking sequences. Nevertheless, this has some disadvantages. The PCR fragments replace either a counter-selectable auxotrophic marker or have to include a selectable marker. In the first case, a counter-selectable auxotrophic marker has to be introduced at the integration site. In the second case, the foreign DNA has to be inserted into a plasmid next to a suitable marker. In both cases, the final yeast strain has to be checked for PCR errors by sequencing the integrated DNA. The newly created integrative plasmids offer something similar to the second case. The integration is restricted to three integration sites, but has the advantage that the sequencing of the integrated DNA can be omitted.

Taken together, the new centromeric, episomal, and integrative plasmids enhance the flexibility of plasmid use in yeast to a great extent. They allow a free choice of growth medium, the use of yeast lacking a suitable auxotrophic marker, and enhance the fidelity of plasmid use.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Christof Taxis, EMBL, Cell Biology and Cell Biophysics Unit, Meyerhofstr. 1, D-69117 Heidelberg, Germany. e-mail: taxis@embl.de