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Gap Repair Transformation in Fission Yeast to Exchange Plasmid-Selectable Markers

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The fission yeast *Schizosaccharomyces pombe* is an attractive model organism for studying eukaryotic biology due to the ease of genetic and molecular manipulations (3). In recent years, several selectable markers and promoters have been developed to enhance our ability to manipulate gene expression in this yeast. Recently, Invitrogen (Carlsbad, CA, USA) has developed the SpECTRA™ *S. pombe* Expression System that consists of three TOPO® cloning vectors that allow for rapid cloning of PCR products, with the option to epitope-tag the gene product (adding a carboxy-terminal V5-6his tag). Transcription of cloned open reading frames is under the control of the thiamine-repressible promoters *nmt1*, *nmt41*, and *nmt81*. (Promoters *nmt41* and *nmt81* are TATA box mutant derivatives of *nmt1* that allow for different

levels of transcription.) However, as all three plasmids utilize the same selectable marker (simian virus 40-driven *Saccharomyces cerevisiae* *LEU2*, which complements an *S. pombe* *leu1* mutant allele), use of these vectors limits the choice of host strains and the possibility to transform strains with more than one plasmid construct.

To enhance the use of the SpECTRA vectors by allowing for the transformation of *Leu*⁺ strains or for the introduction of two distinct plasmids into a host strain, we have developed a protocol that facilitates the exchange of the *LEU2* marker for another selectable marker. This protocol takes advantage of the robust homologous recombination machinery found in yeasts, which has been used in the past to rescue chromosomal mutations onto plasmids by gap repair (8) or to screen mutant alleles created by PCR by co-transformation of the PCR product with a linearized plasmid (5,6). In both situations, the plasmid DNA is linearized before transformation and is repaired and recircularized by DNA that bears homology to the ends of the linearized plasmid. Using a protocol described here, we can readily replace the *LEU2*-selectable

Figure 1. Schematic diagram of SpECTRA plasmid. The three SpECTRA plasmids differ only with in the TATA box of the *nmt1*, *nmt41*, and *nmt81* promoters. The regions homologous to the PCR oligonucleotides used for the marker exchange are indicated as "For" and "Rev". Restriction sites that are unique to this region, and thus suitable for gapping the plasmid within the *LEU2*-selectable marker, are indicated. Also shown are schematics of the PCR products carrying either the *his3* gene (containing a unique *EcoRI* site indicated) or the *ura4* gene (containing two *EcoRI* sites). Not indicated are two *EcoRI* sites in the SpECTRA plasmid that flank the cloning site.

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Table 1. Sequence of Marker Swap PCR Oligonucleotides (5'→3')

<i>LEU2</i> to <i>ura4</i> forward
CTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCGCTTAGCTACAAATCCCCTAGG
<i>LEU2</i> to <i>ura4</i> reverse
GCACATTTCCCCGAAAAGTGCCACCTGAAGCTCAATTCGCCCTTTAGATGTATATGTGCCTTCTGACATAAAACGCCTAG
<i>LEU2</i> to <i>his3</i> forward
CTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCAGATCTCTTTCAACGTTTTCT
<i>LEU2</i> to <i>his3</i> reverse ^a
GCACATTTCCCCGAAAAGTGCCACCTGAAGCTCAATTCGCCCTTTAGATGTATATGAGATCTTCTATGC AAAA GCTAACG
Each oligonucleotide possesses 53 or 56 nucleotides of sequence homologous to the pNMT vectors at the 5'-end, followed by 21–24 nucleotides (underlined) specific to priming a PCR reaction using a <i>ura4</i> (pUC8- <i>ura4</i> ; see Reference 1) or <i>his3</i> (pAF1; see Reference 7) template.
^a The <i>LEU2</i> to <i>his3</i> reverse oligonucleotide possesses a string of four As (in bold) that is present in plasmid pAF1 (7) but not in the genomic sequence of <i>his3</i> , which has only three As at this position. As such, this oligonucleotide is specific to amplification of pAF1 template DNA, while the <i>ura4</i> -specific oligonucleotides should be appropriate for any <i>ura4</i> -containing template.

marker in a SpECTRA vector with either the *S. pombe ura4* or *his3* genes. This technique could be used with other selectable markers as well.

The general procedure is as follows. Marker replacement is carried out by co-transforming *S. pombe* cells with a SpECTRA-derived plasmid carrying an insert of interest that has been digested with restriction enzymes that cut within the *LEU2* gene (Figure 1) along with a PCR product that carries a different selectable marker. The PCR product is flanked by sequences identical to sequences flanking *LEU2* in the SpECTRA vectors (Table 1). Transformants are pooled and subjected to “smash and grab” (4) to rescue autonomous plasmids back into *E. coli*. Plasmids from these *E. coli* transformants are then screened to identify the desired construct.

The oligonucleotides used in this study were designed as follows. The 5'-ends of the forward oligonucleotides contain 53 nucleotides of sequence identical to a region immediately 5' to the unique *NcoI* restriction site in the SpECTRA vectors, while the 5'-ends of the reverse oligonucleotides contain 56 nucleotides of sequence identical to the complement strand immediately 3' to the unique *NsiI* site in these vectors (Figure 1 and Table 1). The 3'-ends of each oligonucleotide set contain 21–24 nucleotides of sequence able to prime a PCR that could amplify either the *S.*

Table 2. Rescue Protocol for Gap-Repaired Plasmids

1. Once colonies have formed on the *S. pombe* transformation plate (after 4–5 days incubation), add 1 mL sterile water to the plate, and collect the cells by gentle scraping with a glass spreader. Transfer to a microcentrifuge tube, and concentrate by a brief centrifugation at 16 000× *g*.
2. Resuspend cell pellet in 200 µL “smash and grab” buffer (10 mM Tris, pH 8.0, 1% SDS, 2% Triton® X-100, 100 mM NaCl, 1 mM EDTA), 200 µL phenol:chloroform, and 300 µL acid-washed glass beads. Lyse cells by vortex mixing for 5 min. Greater than 50% of the cells should be lysed, as observed by microscopy.
3. Pellet cell debris by microcentrifugation at 16 000× *g* for 5 min and remove 50 µL of the top (aqueous) layer to a new microcentrifuge tube. Material close to the interface should be avoided, as it may be contaminated with phenol:chloroform.
4. Add 50 µL isopropanol and precipitate on ice for 10 min, followed by microcentrifugation at 16 000× *g* for 10 min. Remove the liquid, and air-dry the pellet before resuspending in 5 µL sterile water.
5. Use 1 µL DNA preparation to transform electroporation-competent *E. coli* strain XL1-Blue® (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions (although we use 10 µL competent cells in 89 µL sterile water for electroporation and plate the entire transformation onto a single LB Amp plate). This protocol typically yields 10–50 ampicillin-resistant colonies per transformation.

pombe ura4- or the *his3*-selectable marker (for *his3*, the oligonucleotides were designed specifically for plasmid pAF1; see Reference 7). PCR was carried out using the FailSafe™ PCR System (EPICENTRE, Madison, WI, USA), although the specific system used for PCR is most likely not critical to the success of the procedure. The PCR products were 1528 bp and 2150

bp for the *ura4* and *his3* reactions, respectively (Figure 1).

For the marker exchange to occur during yeast transformation, the plasmid must be linearized or gapped by restriction digestion within the *LEU2* region. The PCR product that will be used to repair the gap possesses ends that are homologous to the vector immediately adjacent to unique *NcoI* and *NsiI* sites in

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the plasmid (Figure 1). However, one cannot use restriction enzymes that also cut within the DNA that was cloned into the SpECTRA vector before carrying out the marker swap. As indicated in Figure 1, in addition to *NcoI* and *NsiI*, *StuI*, *BstEII*, *BstBI*, *BtgI* (*DsaI*), *XcmI*, *BspMI*, and *AgeI* all cut the vector specifically within this region. (Complete sequence and restriction map information for these vectors can be found at http://www.invitrogen.com:80/content/vectors/pnmt1topo_seq.txt and http://www.invitrogen.com:80/content/vectors/pnmt1topo_rest.htm.) We have successfully swapped the selectable marker in a pNMT41 plasmid derivative that had been gapped either with *NcoI* and *NsiI* digestion or with *NcoI* and *XcmI* digestion (data not shown). As *XcmI* cuts approximately 0.9 kb from the region of homology present within the PCR product, we infer that any of the enzymes mentioned above can be used to create a gapped vector suitable for marker exchange.

The gap repair transformation was carried out using approximately 2 µg digested plasmid DNA (10 µL volume) along with 2 µg PCR product (10 µL volume) to co-transform either strain FWP16 (*h⁺ ura4-D18*) to *Ura⁺* or strain JSP303 (*h⁺ leu1-32 his3-D1*) to *His⁺*, plating transformants onto PM-uracil or PM-histidine (9) medium, respectively. The transformation was performed using the protocol of Bähler et al. (2), which enhances homologous recombination events. In a control transformation, only the PCR product was added to competent cells to determine the level of colony formation due to nonhomologous integration of the PCR product or to uptake and unstable maintenance of the PCR product. Transformation with PCR product alone gave a background of several hundred colonies, while co-transformation of the PCR product with the gapped plasmid resulted in greater than 1000 colonies after four days at 30°C. The background level of *His⁺* or *Ura⁺* colonies generated by events other than plasmid gap repair does not pose a problem for the identification of the desired plasmids since these colonies do not contain DNA that can transform *E. coli* to ampicillin-resistance.

Gap-repaired plasmids were rescued

from *S. pombe* transformants into *E. coli* using a modified version of the "smash and grab" method (4), as described in the protocol in Table 2. Following this procedure, we typically observe 10–50 *E. coli* transformants. Restriction mapping of candidate plasmids demonstrated that 80%–100% of *E. coli* transformants carry the desired plasmid. *EcoRV* can be used to identify *ura4*-containing plasmids, as this enzyme cuts once within *ura4* while not cutting within *LEU2* (Figure 1). Additionally, *HindIII* cuts once within *LEU2* while not cutting within *ura4*. *EcoRI* can be used to identify *his3*-containing plasmids, as this enzyme generates a 0.55-kb fragment internal to *his3* (Figure 1). Finally, we have shown that the purified plasmids can transform *ura4⁻* or *his3⁻* mutant strains to *Ura⁺* or *His⁺*, respectively (data not shown).

Using the protocol described here, we have been able to convert the *LEU2*-marked SpECTRA plasmids quickly and efficiently to either *ura4⁻* or *his3⁻*-marked plasmids. This strategy can be easily adapted for the incorporation of other selectable markers by modifying the gene-specific sequence (3'-end, underlined in Table 1) of the PCR oligonucleotides combined with an appropriate template DNA. This general technique could also be used for marker exchange in other *S. pombe* or *S. cerevisiae* shuttle vectors in situations where a lack of appropriate restriction sites precludes standard subcloning procedures. The ease with which plasmid markers can be exchanged by gap repair transformation greatly enhances the usefulness of the SpECTRA cloning vectors by allowing for transformation of *Leu⁺* host strains or for the co-transformation of host strains with SpECTRA-derived constructs that express two different proteins.

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