Short Communication

Marker-free insertion of a series of C-terminal epitopes based on the 50:50 method in Saccharomyces cerevisiae

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Masamichi Nonaka and Tsutomu Kishi*

College of Engineering, Nihon University, Koriyama, Fukushima 963-8642, Japan

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Saccharomyces cerevisiae is a popular model organism for biological studies. Accordingly, the molecular mechanisms of various cellular processes have been characterized in S. cerevisiae, many of which have been found to be widely conserved among different species. These discoveries have mainly been possible owing to the availability of powerful genetic tools for S. cerevisiae. One important tool is the gene modification technique, in which a transformation DNA construct with a selectable marker can be integrated into a desired locus by homologous recombination (Rothstein, 1991). With the development of the one-step polymerase chain reaction (PCR)-mediated methods (Baudin et al., 1993; Lorenz et al., 1995; McElver and Weber, 1992), gene modifications, including gene deletion, replacement of an endogenous promoter with a regulatable promoter, and insertion of epitope tags, have now become convenient (Longtine et al., 1998; Wach et al., 1994, 1997). However, the application of this technology for multiple replacements is sometimes inconvenient because of the limitation of available markers.

To overcome this limitation, various strategies for marker recycling have been proposed, by which the same marker can be used repeatedly. In principle, marker recycling involves replacing DNA sequences used as selection markers with those derived from foreign sequences such as the bacterial hisG or loxP site (Alani et al., 1987; Gülde et al., 1996; Nihei and Kishi, 2017). However, accumulation of such sequences in a single cell may not be preferable, because it could inhibit correct replacement in the subsequent transformation or cause gene rearrangements (David and Siewers, 2015). An alternative approach is to conduct gene replacement without foreign DNA sequences (Akada et al., 2006; Carvalho et al., 2013; Horecka and Davis, 2014; Langle-Rouault and Jacobs, 1995; Stori et al., 2001). The simplest example is the so-called 50:50 method (Horecka and Davis, 2014), which allows for gene deletion without a heterologous sequence via a single PCR step, followed by transformation and subsequent counter-selection. The outstanding feature of this method is the use of 50:50 primers that are designed to direct a pop-out recombination event just downstream of the upstream pop-in site. The heterologous sequences introduced by pop-in recombination are then excised by the pop-out recombination, leaving no traces. This method is also applicable for the insertion of a codon, site-directed mutation, as well as deletion of two nucleotides for introducing a frameshift mutation (Horecka and Davis, 2014). However, there is currently no method for epitope tagging based on the 50:50 strategy.

Here, we have constructed a new set of plasmids for the C-terminal tagging of epitopes with no extraneous sequences other than the epitopes on the basis of the 50:50 method. The modules used for PCR-mediated epitope tagging are shown in Fig. 1A. Starting from the plasmid pF6a-3xHA-His3MX6 (Longtine et al., 1998), which is widely used for the insertion of the 3xHA epitope, two modifications were introduced. First, we replaced the His3MX6 marker with the GAL10p-GIN1M86-LEU2 cassette (Akada et al., 2002) to introduce a counter-selection marker, resulting in plasmid pMN-12. The GAL10p-GIN1M86 DNA sequence inhibits cell growth when overexpressed from the GAL10 promoter (Akada et al., 1998), which is widely used for the insertion of the 3xHA epitope, two modifications were introduced. First, we replaced the His3MX6 marker with the GAL10p-GIN1M86-LEU2 cassette (Akada et al., 2002) to introduce a counter-selection marker, resulting in the plasmid pMN-12. The GAL10p-GIN1M86 DNA sequence inhibits cell growth when overexpressed from the GAL10 promoter (Akada et al., 2002). Therefore, pop-out recombinants can be selected in medium that contains galactose as a carbon source. LEU2 is used as a transformation marker. Second, to expand the utility of the plasmid, we replaced the DNA sequence encoding the 3xHA epitope in pMN-12 with those for dual tags in which a single HA epitope was tagged with each of 13xMyc and 3xFLAG, resulting in the plasmids pMN-13 and pMN-15, respectively (Fig. 1A, and see below).
Details on plasmid construction are provided in Supplementary Information. Primers used in the present study are shown in Table 1. In contrast to the original 50:50 method, we used 40:40 primers in this new system. We routinely performed pop-in recombination for gene modification with primers of 40-nucleotide homology at the target locus, which was also found to be sufficient for pop-out recombination, as described below. The guidelines for primer synthesis are shown in Fig. 1B. As indicated above, one of the key merits in using the 50:50 method is the insertion of epitopes with no other extraneous sequences. Such insertion can be attained if the reverse primers (in our system, 40:40 primers) are designed to direct pop-out recombination at DNA sequences coding for the C-terminal tags. For example, to tag the 3xHA at the 3'-end of the SWI5 gene, which encodes a transcription factor for Sic1 that inhibits S-phase entry (Kishi et al., 2008; Knapp et al., 1996), the reverse 40:40 primers should be designed to direct a pop-out recombination event at the last repeat in the triple tandem HA sequences (Fig. 1C). Therefore, the reverse 40:40 primers should contain the 40-nucleotide sequence that is complementary to the coding sequence of the third HA repeat in the 3xHA sequences. This fragment is then used to obtain pop-in recombinants. The ADH1-11p-GAL10p-GIN11M86-LEU2 sequence is excised by pop-out recombination at the homologous HA repeats that are located upstream and downstream of GAL10p-GIN11M86, which results in the production of SWI5-3xHA cells. Arrows indicate the positions of primers for examination of the correct gene replacements. (D) 3xHA DNA coding sequence. Bold letters indicate the target sequence for pop-out recombination. A BamHI site is also shown.

Table 1. Primers used in the present study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5'-3')</th>
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<tbody>
<tr>
<td>#31</td>
<td>GGTCTTCAGTGCTCACAATCAAAG</td>
</tr>
<tr>
<td>#226</td>
<td>AACGGGAAGATGTTTTCGCCATGAAAAAAACTAAACAAAGGCGATCCCCGGTTATAAA</td>
</tr>
<tr>
<td>#2376</td>
<td>CCACACATTCCCATCCTTCG</td>
</tr>
<tr>
<td>#3565</td>
<td>TGTATTAAATATATAAATGAGTTCCATAACACATACAGGTTTTCGCCATGAAAAGCGATTTGTCAGAGCC</td>
</tr>
<tr>
<td></td>
<td>GAATCTGGAAACGTCTATAGGAGAGGGGAATTTCAGGTCGTCTTAAAC</td>
</tr>
</tbody>
</table>

Fig. 1. Modules for PCR-mediated epitope tagging and the design of their primers. (A) Modules in the plasmids pMN-12, pMN-13, and pMN-15 for the PCR-mediated insertion of C-terminal tags. The locations of 3xHA, 13xMyc-HA, 3xFLAG-HA, ADH11p (ADH1 terminator), GAL10p-GIN11M86, and LEU2 are shown. The positions of the first, second, and third HA sequences are also designated as 1st, 2nd, and 3rd, respectively. These modules are cloned in the pFA6a backbone. The positions of the forward (F2) and reverse 40:40 primers are also shown. BamHI in the HA sequence is lost during the construction of pMN-13 and pMN-15. (B) Standard design for the forward and reverse 40:40 primers. The same primer pairs can be used to amplify all of the modules in (A). (C) Schematic representation of the C-terminal insertion of 3xHA in the SWI5 locus. A DNA fragment amplified by PCR with pMN-12 as a template using the appropriately designed primers contains 3xHA, ADH11p-GAL10p-GIN11M86-LEU2, and HA sequences. This fragment is then used to obtain pop-in recombinants. The ADH11p-GAL10p-GIN11M86-LEU2 sequence is excised by pop-out recombination at the homologous HA repeats that are located upstream and downstream of GAL10p-GIN11M86, which results in the production of SWI5-3xHA cells. Arrows indicate the positions of primers for examination of the correct gene replacements. (D) 3xHA DNA coding sequence. Bold letters indicate the target sequence for pop-out recombination. A BamHI site is also shown.
polymerase (New England Biolabs) was used for PCR according to the manufacturer’s recommendations.

We next validated our strategy of epitope insertion. We first fused the 3xHA sequence with the chromosomal SWI5 locus. In the first step, cells with the SWI5-3xHA-ADH1-GAL10p-GIN11M86-LEU2-HA module were constructed. TK-997 cells (Mata, bar1::TRP1, ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3-52, can1-100) were transformed with a DNA fragment amplified by PCR using pMN-12 as a template with primers #226 and #3565, and cells with a DNA fragment amplified by PCR using pMN-12 as a template with primers #226 and #3565, and cells with the Leu+ phenotype were selected on SD-Leu plates (0.17% yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 2% glucose, 2% agar, and appropriate amino acids but lacking leucine) (Fig. 2A). Yeast transformation was performed as described by Gietz and Schiestl (2007). The expected insertion of the 3xHA-ADH1-GAL10p-GIN11M86-LEU2-HA module at the C-terminal end of SWI5 was verified by genomic PCR with the forward primer #31, which anneals to the precise tagging of 3xHA with no other extraneous sequences at the 3'-end of SWI5, and the reverse 40:40 primer #3565 (Fig. 2B). The positions of PCR primers are shown in Fig. 1C. Genomic DNA was prepared as described previously (Hoffman and Winston, 1987).

We next selected pop-out recombinants. Cells that excised the ADH1-GAL10p-GIN11M86-LEU2 module should grow on YPRG plates (1% yeast extract, 2% peptone, 2% raffinose, 2% galactose, and 2% agar) but show the Leu phenotype. We therefore cultured a single colony of the SWI5-3xHA-ADH1-GAL10p-GIN11M86-LEU2-HA cells in YPD liquid medium (1% yeast extract, 2% peptone, and 2% glucose) for one day, and then aliquots were taken, spread on YPRG plates, and the plates were incubated at 30°C for two days. The growth of the colonies was assessed on SD-Leu plates. Among the 20 colonies that were picked up from the YPRG plates, 12 showed no growth on the SD-Leu plates; Fig. 2A shows representative images of such clones. To further confirm the expected excision, genomic PCR was performed for the 12 clones with the reverse primer #2376, which anneals downstream of SWI5, and the forward primer #31 (Fig. 1C). A DNA fragment with the expected size was detected in all 12 clones, indicating loss of the ADH1-GAL10p-GIN11M86-LEU2 module (Fig. 2B). Genomic sequencing confirmed the precise tagging of 3xHA with no other extraneous sequences at the 3'-end of SWI5. No mutations that could be attributed to errors in oligonucleotide synthesis or PCR were observed. No transformants were obtained for which the pop-out recombination occurred at the first or second positions of PCR primers are shown in Fig. 1C. Genomic DNA was prepared as described previously (Hoffman and Winston, 1987).

We next selected pop-out recombinants. Cells that excised the ADH1-GAL10p-GIN11M86-LEU2 module should grow on YPRG plates (1% yeast extract, 2% peptone, 2% raffinose, 2% galactose, and 2% agar) but show the Leu phenotype. We therefore cultured a single colony of the SWI5-3xHA-ADH1-GAL10p-GIN11M86-LEU2-HA cells in YPD liquid medium (1% yeast extract, 2% peptone, and 2% glucose) for one day, and then aliquots were taken, spread on YPRG plates, and the plates were incubated at 30°C for two days. The growth of the colonies was assessed on SD-Leu plates. Among the 20 colonies that were picked up from the YPRG plates, 12 showed no growth on the SD-Leu plates; Fig. 2A shows representative images of such clones. To further confirm the expected
the 40-nucleotide sequence is sufficient for pop-out recombination. Expression of Swi5-3xHA was also examined by immunoblotting. A single colony of each of the SWIS-3xHA, SWIS-13xMyc-HA, and SWIS-3xFLAG-HA cells was cultured in YPD liquid medium to an OD_{600} of ~0.8, and then 5 mL of the cultures was harvested and lysed as described by Kishi et al. (2008), electrophoresed on 5–20% gradient gels (Wako), and immunoblotted with anti-HA (12CA5) antibody. The anti-HA-interacting moieties were detected with molecular masses of 90–100 kDa, corresponding to those of Swi5-HA and its phosphorylated forms (Fig. 3). Taken together, these results confirmed the successful insertion of C-terminal 3xHA tags with no other extraneous DNA sequences using the plasmid pMN-12.

Similarly, we inserted 13xMyc-HA and 3xFLAG-HA tags using pMN-13 and pMN-15 with the same primer pair (#226 and #3565). Pop-in and pop-out recombinants were selected as described above, and the results are shown in Figs. 2A and B. Marker-free insertion of each tag was confirmed by genomic sequencing, and western blot analysis with anti-Myc (9E10) and anti-FLAG (M2) antibodies demonstrated the expression of Swi5-13Myc-HA and Swi5-3xFLAG-HA (Fig. 3).

In summary, we have established a new method for the PCR-mediated insertion of C-terminal tags (3xHA, 13xMyc-HA, and 3xFLAG-HA) in *S. cerevisiae* by constructing the plasmids pMN-12, -13, and -15. The same primer pairs can be used to amplify tags in all of these plasmids. The most outstanding feature of this method is that it enables the insertion of tags without markers and other heterologous sequences. Such insertions are simply achieved via reverse primers designed to direct a pop-out recombination event at the HA-coding sequences. This strategy is based on the 50:50 method, with a modification of using a 40:40 primer. Our results indicated that 40-nucleotide homology is sufficient for precise pop-out recombination, allowing for more economical primer synthesis. In addition, our proposed method can be applied to any transformation marker, which is an advantage over the 50:50 method where URA3 is the inevitable selection marker. Another advantageous feature of this method is its simplicity, requiring only a single PCR, single yeast transformation step, and subsequent selection with clones that grow with galactose but exhibit the Leu-phenotype. Furthermore, it could be possible to introduce a new gene tag using pMN-12, -13 and -15 into cells that already carry HA tags. Indeed, use of the resulting cells has an advantage of detecting different proteins simultaneously with anti-HA antibody. However, since such cells cannot be applied for co-immunoprecipitation with anti-HA antibody, construction of new plasmids with a single epitope tag other than HA would be required. Finally, we believe that the new proposed method could be a powerful tool for yeast genetics in the future.

Acknowledgments

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Supplementary Materials

Supplementary information is available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

References


