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Systematic targeted gene deletion using the gene-synthesis method in fission yeast

Miyoung Nam [a,1], Sook-Jeong Lee [b,1], Sangjo Han [c,1], Dongsup Kim [c], Minho Lee [d], Eun-Jung Kang [e], Han-Oh Park [e], Ah-Reum Lee [b], Sol Lee [b], Cheol-Hee Kim [a], Dong-Uk Kim [f,*], Kwang-Lae Hoe [b,*]

[a] Department of Biology, Chungnam National University, Yuseong, Daejeon 305-764, Republic of Korea
[b] Department of New Drug Discovery and Development, Chungnam National University, Yuseong, Daejeon 305-764, Republic of Korea
[c] Bioinformatics Lab, Healthcare Group, SK Telecom, Sungnam, Kyunggi, 463-784, Republic of Korea
[d] Department of Biological Science, Sangji University, Wonju, Gangwon 220-702, Republic of Korea
[e] Bioneer Corporation, Daedeok, Daejeon 306-220, Republic of Korea
[f] Aging Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Yuseong, Daejeon 305-764, Republic of Korea

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ABSTRACT

Genome-wide targeted gene deletion, a systematic method to study gene function by replacing target genes with deletion cassettes, using serial-PCR or block-PCR requires elaborate skill. We developed a novel gene-synthesis method to systematically prepare deletion cassettes on a 96-well basis in fission yeast. We designed the 2129-bp deletion cassette as three modules: a central 1397-bp KanMX4 selection marker module and two flanking 366-bp gene-specific artificial linker modules. The central KanMX4 module can be used in multiple deletion cassettes in combination with different sets of flanking modules. The deletion cassettes consisted of 147 oligonucleotides (93 for the central module + 25 for each of the flanking modules + 4 for the joints) and the oligonucleotides were designed as ~29 mers using an in-house program. Oligonucleotides were synthesized on a 96-well basis and ligated into deletion cassettes without gaps by ligase chain reaction, which was followed by two rounds of nested PCR to amplify trace amounts of the ligated cassettes. After the artificial linkers were removed from the deletion cassettes, the cassettes were transformed into wild-type diploid fission yeast strain SP286. We validated the transformed colonies via check PCR and subjected them to tetrad analysis to confirm functional integrity. Using this method, we systematically deleted 563 genes in the fission yeast *Schizosaccharomyces pombe* with a >90% success rate and a point-mutation rate of ~0.4 mutations per kb. Our method can be used to create systematic gene deletions in a variety of yeasts especially when it included a bar-code system for parallel analyses.

1. Introduction

Gene-targeted deletion is a popular technology that knocks out a gene by replacing it with a selection marker via homologous recombination. Eukaryotic gene targeting was first reported in yeasts in 1982 (Shortle et al., 1982) and has since been applied to various model organisms such as drosophila (Rong and Golic, 2000), nematodes (Fraser et al., 2000; Piano et al., 2000), and mice (Muller et al., 1994; Ramirez-Solis et al., 1993). The systematic knock-out of whole genes, a gene-by-gene removal procedure that generates a comprehensive library of single-gene deletions, has been conducted in budding and fission yeasts (Giaever et al., 2002; Kim et al., 2010; Shoemaker et al., 1996) and drosophila (Rong and Golic, 2000). In nematodes, systematic knock-down by an RNAi system has been introduced for selective gene targeting (Fraser et al., 2000; Piano et al., 2000). Systematic gene-deletion studies have provided insights into functional genomics, proteomics, phenomics, and drug targeting (Han et al., 2013; Lum et al., 2004).

Systematic gene deletion in yeasts requires a specialized DNA fragment called a deletion cassette, which generally consists of a central selection marker and a pair of flanking chromosomal homologous regions (CHRs). In addition, a pair of bar-code sequences is usually included for parallel analyses (Giaever et al., 2002; Kim et al., 2010). The success rate of a gene deletion for a given species increases with the length of the CHRs (Decottignies et al., 2003; Manivasakam et al., 1995), but the advantage of increasing the length of the CHRs can be compromised by a corresponding increase in the difficulty of cassette preparation. In the budding yeast *Saccharomyces cerevisiae*, deletion

Abbreviations: CHR, chromosomal homologous region; LCR, ligase chain reaction; ORF, open reading frame.

* Corresponding authors at: Department of New Drug Discovery and Development, Chungnam National University, 99 Daehak-ro, Yuseong, Daejeon 305-764, Republic of Korea. Tel.: +82 42 821 8627; fax: +82 42 821 8927.
E-mail addresses: Kimdongu@kribb.re.kr (D.-U. Kim), Kwanghoe@cnu.ac.kr (K.-L. Hoe).

[1] These authors contributed equally to this work.

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cassettes containing pairs of ~50-bp CHRs were prepared by several rounds of serial PCR and worked for most targets (Giaever et al., 2002; Winzeler et al., 1999). In the fission yeast *Schizosaccharomyces pombe*, however, 31% of the deletion library (1,515 of 4,836 targets) was constructed by the same method (Kim et al., 2010), and the rest of the targets required an increase in the length of the CHRs. In fact, more than two thirds of all the targets (3321 of 4836 targets) were constructed using ~250-bp CHRs, which were prepared by block PCR (Bahler et al., 1998; Decottignies et al., 2003; Gregan et al., 2005; Rothstein, 1983). The block PCR required the purification of PCR-amplified DNA fragments by gel elution, which was elaborate and required great technical skill. In order to replace the elaborate gel-elution step with an automated procedure, we previously developed a novel gene-synthesis method to create systematic gene deletions (Kim et al., 2010), which despite its convenience, still needed readjusting and fine tuning to achieve a maximum success rate.

Here, we describe the optimization as well as the critical strategy of the gene-synthesis method for creating gene-deletion libraries in fission yeast. Our method of creating systematic gene deletions by gene synthesis on a 96-well basis is potentially applicable to a variety of yeasts.

2. Methods

2.1. Chemicals, enzymes, and strains

All the oligonucleotides used in this study were custom synthesized by Bioneer Corporation (Daejeon, Korea) and supplied on a 96-well basis for the sake of convenience. All enzymes and assay kits including T4 polynucleotide kinase (catalogue no. E-3111), thermo-stable *Tfi* DNA ligase (catalogue no. E-3111), *Pfu* polymerase (catalogue no. E-2015), and PCR premix (catalogue no. K-2012) were supplied by Bioneer. Restriction enzyme *Eco*31I was purchased from Fermentas (Waltham, MA, USA). The diploid fission yeast *S. pombe* strain SP286 (*ade6-M210*/*ade6-M216*, *ura4-D18*/*ura4-D18*, *leu1-32*/*leu1-32h*$^{+}$/*h*$^{+}$) was used as a host for the systematic gene deletions (Kim et al., 2010). Culture media for the yeast were obtained from BD Biosciences (San Jose, CA, USA) and used according to the manufacturer's instructions. Kanamycin (G418) was purchased from Duchefa Biochemie (catalogue no. G0175, Haarlem, Netherlands). General chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

2.2. Design of the deletion cassette

The 2129-bp deletion cassette was divided into three modules consisting of a 1397-bp invariable kanamycin-resistance (KanMX4) module and a pair of variable 366-bp flanking gene-specific modules (Fig. 1A). The KanMX4 sequence was derived from Tn903 of *Escherichia coli*, and its promoter and terminator were derived from those of *TEF* in the yeast *Ashbya gossypii*. The gene-specific 5′ and 3′ CHRs (each with a length of 250 bp) were derived from the promoter region upstream of the ATG start codon and the terminator region downstream of the stop codon(s), respectively, of the target gene.

The 46-bp artificial linker included two pairs of primer sites for two rounds of nested PCR (L1-F/L1-R and L2-F/L2-R) and the *Eco*31I restriction site (5′-GGTCTCN-3′) for the removal of the primer sites (Fig. 1B). The 70-bp bar-code region was designed to contain a central up-tag or down-tag with flanking gaps and universal primer sites for PCR amplification of the bar codes in parallel analyses (Fig. 1C). The sequences of the artificial linkers and bar-code regions were generated as previously reported (Kim et al., 2010).

2.3. Software for oligonucleotide design

To design the entire oligonucleotide set for a deletion cassette, we used BatchBlock2Oligo (http://pombe.kaist.ac.kr/block2oligo/batch_block2oligo.pl), merged from Block2Oligo and iBlocksAssembly, the two algorithms used in our previous study (Kim et al., 2010). We constructed 263 deletion cassettes using the two older algorithms separately and another 300 deletion cassettes using the new merged program. Basically, the design of oligonucleotides was optimized to minimize Tm differences. A Tm of 60 ± 3 °C was considered as a first parameter, with the Tm calculated following the nearest-neighbor method (SantaLucia, 1998). Also, an oligonucleotide length of 25–35 mer was considered an important factor to minimize errors in chemical synthesis.

2.4. Ligase chain reaction (LCR)

To assemble all the oligonucleotides into a single deletion cassette without any nicks, LCR was performed on a 96-well basis using the thermo-stable DNA ligase *Tfi* in repeated ligation reactions. Prior to the LCR, the 5′ ends of all the oligonucleotides (20 picomole each oligonucleotides) were phosphorylated using T4 polynucleotide kinase and ATP at 37 °C for 5 h, followed by an additional 5-h incubation after the addition of extra kinase. Half of the total phosphorylated product was subsequently subjected to 40 cycles of LCR, with each cycle consisting of 5-min denaturation at 95 °C and 5-min ligation at 54–59 °C.

2.5. Nested PCR using the artificial linkers and assessment of the point-mutation rate

Because extremely trace amounts of deletion cassettes without nicks were generated by the LCR, the cassettes had to be amplified by two consecutive rounds of PCR using the L1-F/L1-R and L2-F/L2-R primer pairs (Fig. 1B). The nested PCR was performed under the following conditions: 20 μl total volume containing 50 picomole each L1-F/L1-R or L2-F/L2-R primer pair, 2.5 U *Pfu*, and 0.25 mM dNTP; 35 reaction cycles consisting of 30-s denaturation at 94 °C, 30-s annealing at 60 °C, and 2-min polymerization at 72 °C. The amplified deletion cassettes were subjected to dideoxy sequencing using the linker regions as primer sites. The point-mutation rate among more than 100 deletion cassettes was assessed and compared with those obtained using previous methods (Smith et al., 2003; Xiong et al., 2004).

2.6. Transformation with deletion cassettes, check PCR, and tetrad analysis

The artificial linkers were removed using *Eco*31I after PCR amplification and before transformation, because they could interrupt homologous recombination, yielding low success rates. The deletion cassettes were transformed into SP286 cells using the lithium acetate method (Moreno et al., 1991). Transformed colonies were incubated on YES agar plates containing 200 μg/ml G418 at 30 °C for 3–4 days. Positive colonies were selected and subjected to a check PCR to verify the insertion of the deletion cassettes at the correct loci. Briefly, a single colony grown on YES agar plates was suspended in distilled water, and then subjected to several rounds of boiling. The suspended solution was used for the check PCR with the PCR premix and appropriate sets of primer pairs as previously reported (Kim et al., 2010). Positive colonies were confirmed by running the PCR product on agarose gel. After the check PCR, tetrad analysis was performed to check whether the cells contained any extra mutations. Briefly, a heterozygous deletion mutant was transformed with pON177 (Styrkarsdottir et al., 1993) (kindly gifted by O. Nielsen) to mimic sexual mating, and the transformed cells were left to germinate for 4–5 days on minimal (+adenine and leucine) plates. Using a microscopic manipulator (Singer MSM, Somerset, England), the spores were dissected on YES plates for 4–5 days at 30 °C. The segregation pattern and growth profiles of the spores were carefully examined for G418 sensitivity and any secondary mutations (Kim et al., 2010).