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Notes & Tips

An efficient site-directed mutagenesis method for ColE1-type *ori* plasmid

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Polymerase chain reactions (PCR) coupled with mutagenic primer-based site-directed mutagenesis have been extensively employed for probing the structure–function relationship of proteins and/or nucleic acids. Many methods to obtain single-basepair changes, deletions, and insertions have been described during the past decades [1–5]. All of these procedures require single- or double-stranded DNA as a template and one to four primer(s) with the necessary mutation(s). Purification of intermediate reaction products and sequencing of the mutated gene make some of these methods complicated and time consuming. A single inverse PCR with two primers method for site-directed mutagenesis is very simple and rapid [5], but the practical application is generally limited to the relatively small-sized plasmid and depends largely on efficiency and fidelity of the polymerase.

Every plasmid contains one or more DNA sequences that function as an origin of replication or *ori* (a starting point for DNA replication). Replication of ColE1-type plasmids is initiated at a unique *ori* site and is unidirectional [6–8]. The origin of ColE1 replication spans a region of about 1 kb that includes several subregion DNA sequences. The replication process of ColE1 depends on RNA II, a plasmid-encoded RNA molecule, and is initiated 555 nucleotides upstream of *ori* [7,9]. Anything that disturbs the formation of the correct secondary structure of RNA II will interfere with the plasmid replication. The replication from the ColE1 *ori* region does not require the associated proteins (e.g., *dnaA* protein) like *oriC* but requires the DNA Polymerase I (PolI) enzyme [10], a RNA polymerase, and RNase H [10–12] from host.

Based on the above mechanism, we adapted the self-ligation of inverse PCR method for site-directed mutagenesis. Fig. 1 shows in a schematic diagram an overview of our mutagenesis protocol. First, two halves of a plasmid were amplified by PCR using four primers, of which a pair of tail-to-tail primers is within the mutant region and another pair of tail-to-tail primers is complementary coding for ColE1-type *ori* region. TaKaRa LA Taq polymerase is chosen as an enzyme for higher efficiency, higher fidelity, and high proofreading capacity. Then, following gel purification, PCR products were treated with blunting and phosphorylation. Next, two fragments of PCR products were ligated. There are many types of circularizing ligation products, but only those that have an intact ColE1-type *ori* region can amplify in *Escherichia coli*.

To test the applicability of this site-directed mutagenesis strategy, we generated a series of HsYif1 mutants. Yif1 is well conserved across species and HsYif1, a Golgi protein cycling between endoplasmic reticulam and Golgi apparatus, interacts with Yip1A [13,14]. To analyze the function of conserved residues within the N terminus of HsYif1 (Fig. 2a), residues L108/109, P112, and W118 were planned to be generated mutants. We performed PCR in a 50 μl volume containing 10 ng of GFP-HsYif1 plasmid template [14], 50 pmol each of forward and reverse primers (Supplementary material, Table S1), 20 nmol dNTP, 5 μl Mg^{2+} plus buffer, and 2.5 U LA Taq polymerase (Takara Biotechnology, Dalian, China). The thermocycler was preheated at 94 °C for an initial denaturation step of 3 min, followed by 5 cycles of 94 °C for 30 s, 48 °C for 30 s, and 72 °C for 4 min, then 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 4 min, and finally 1 cycle of 72 °C for 10 min. The PCR products were agarose

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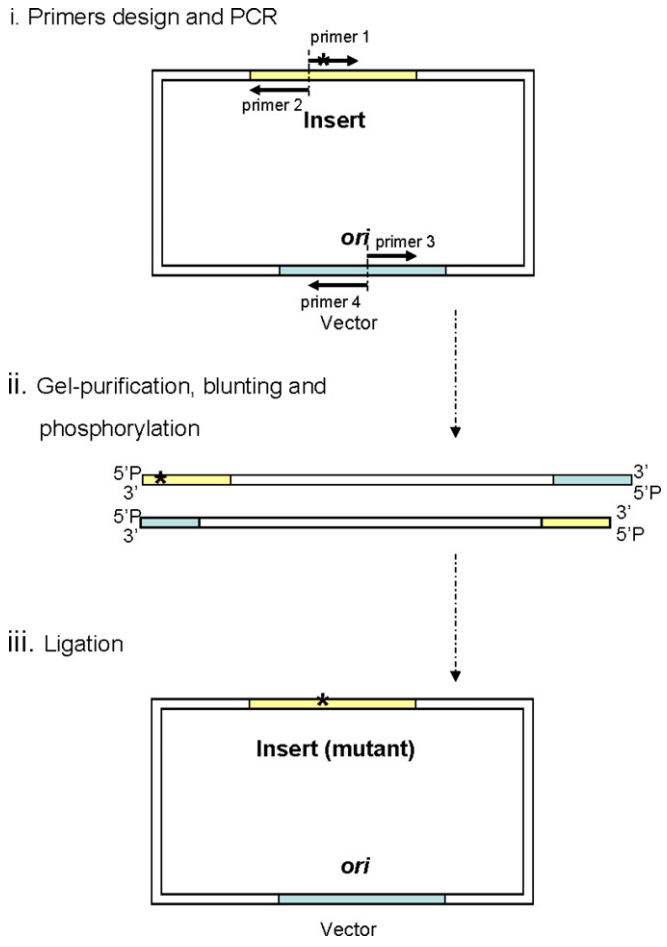

Fig. 1. Diagram of steps of site-directed mutagenesis for ColE1-type *ori* plasmid. Four primers were synthesized for this method, of which a pair of tail-to-tail primers is within the mutant region and another pair of tail-to-tail primers is complementary coding for a part of ColE1-type *ori* region. Two halves of a plasmid were amplified by independent PCR using primers 1 and 3 and primers 2 and 4. After gel purification, blunting, and phosphorylation, two PCR-amplified fragments were joined by ligase. Only the circularizing ligation products which have intact ColE1-type *ori* region and desired mutant can amplify in *E. coli*. *Indicates mutant side.

gel-fractionated and column-purified using a PCR purification kit (Promega). Following blunting and phosphorylation, two fragments of PCR products were ligated at 16 °C for 1 h. The blunting, phosphorylation, and ligation were performed with Takara blunting and kination kit and ligation kit (Takara Biotechnology) according to the manufacturer's instructions. After transforming DH5α *E. coli* bacteria with the ligation mixture, the plasmids were prepared, analyzed by restriction digestion, and randomly selected for sequencing. Sequencing results demonstrated that the plasmids contained the desired mutation without any other alteration from three of three independent site-directed-mutagenesis-experiment-transformed colonies (Fig. 2b). Furthermore, immunofluorescence revealed that the localization of GFP-HsYif1$^{L108/109A}$, GFP-HsYif1^{P112A}, and GFP-HsYif1^{W118A} mutants was different from Golgi localization of GFP-HsYif1 (Fig. 2c), indicating that conserved residues within N terminus of HsYif1 were important for its Golgi localization.

In this report, we describe a site-directed mutagenesis method with four primers, two primers within ColE1-type *ori* region and two primers within mutant region. With this approach, site-directed mutagenesis can be obtained easily and successfully by circularizing two fragments of PCR products together. This method allows carrying mutations more efficiently than that with the traditional mutagenesis methods and single inverted PCR method, according to the statistic data in our laboratory (Supplementary material, Table S2). In addition, mutations such as insertions and deletions can be performed with this method. Moreover, by halving a plasmid into two parts, this method can perform relatively large-sized plasmids and rapidly generate chimera using two different proteins' cDNA cloned in the same vector.

Most cloning plasmids carry the modified ColE1 origin of replication derived from pUC vectors, which deleted the *rop* replication regulatory region of ColE1 origin to maintain a high copy number of the plasmid, or from pBR322 vector, including pET Vectors (Novagen), pGEX Vectors (GE Healthcare), pcDNA Vectors (Invitrogen), and Living Colors Fluorescent Protein Vectors (Clontech Laboratories). Thus, this site-directed mutagenesis method can be practically applied generally and the pair of primers within ColE1-type *ori* region can be used commonly. Moreover, other functional genes, e.g., antibiotic resistance gene, can be used for plasmid mutagenesis selection, based on functional gene selection mechanism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2006.12.007.

Fig. 2. Replacement of amino acids within the N terminus of HsYif1 by site-directed mutagenesis using ColE1-type *ori* plasmid and the localization of wild-type HsYif1 and its mutants. (a) Conserved residues within N terminus of Yif1 across species. Arrows indicate residues mutated. (b) Sequences of mutant regions of GFP-HsYif1 mutants. Arrows indicate the mutant sites in GFP-HsYif1$^{L108/109A}$, GFP-HsYif1^{P112A}, and GFP-HsYif1^{W118A} mutants and corresponding sites in the wild-type GFP-HsYif1. (c) Subcellular distribution of wild-type GFP-HsYif1, GFP-HsYif1$^{L108/109A}$, GFP-HsYif1^{P112A} and GFP-HsYif1^{W118A} mutants in HeLa cell. Bars, 10 μm.

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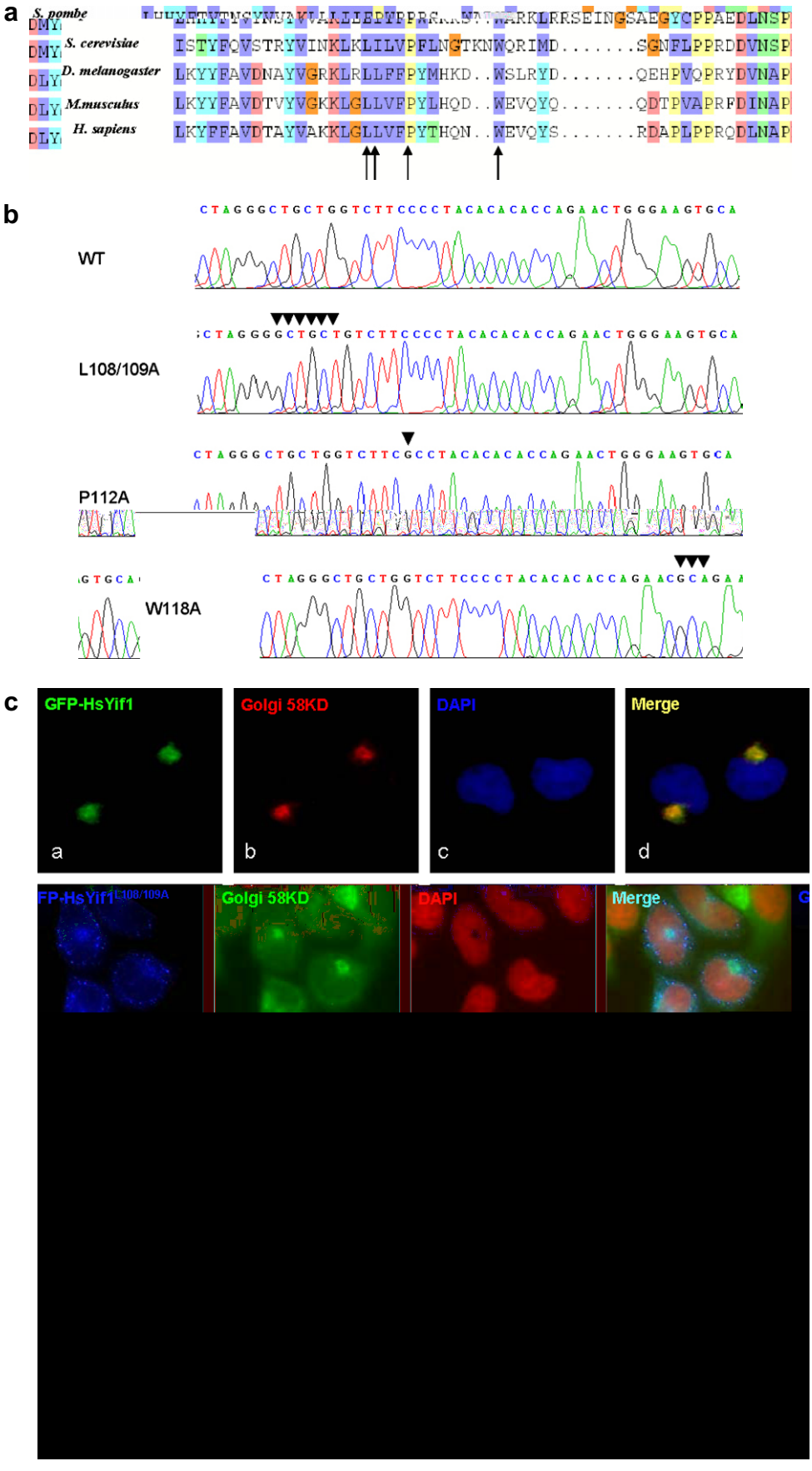
a
S. pombe
S. cerevisiae
D. melanogaster
M.musculus
H. sapiens
b
WT
L108/109A
P112A
W118A
c
GFP-HsYif1
Golgi 58KD
DAPI
Merge
a
b
c
d

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