Published in final edited form as:

Methods Mol Biol. 2011 ; 745: 173–191. doi:10.1007/978-1-61779-129-1_11.

In Vivo Site-Specific Mutagenesis and Gene Collage Using the Delitto Perfetto System in Yeast *Saccharomyces cerevisiae*

Samantha Stuckey, Kuntal Mukherjee, and Francesca Storici

Abstract

Delitto perfetto is a site-specific in vivo mutagenesis system that has been developed to generate changes at will in the genome of the yeast *Saccharomyces cerevisiae*. Using this technique, it is possible to rapidly and efficiently engineer yeast strains without requiring several intermediate steps as it functions in only two steps, both of which rely on homologous recombination to drive the changes to the target DNA region. The first step involves the insertion of a cassette containing two markers at or near the locus to be altered. The second step involves complete removal of this cassette with oligonucleotides and/or other genetic material and transfer of the expected genetic modification(s) to the chosen DNA locus. Here we provide a detailed protocol of the *delitto perfetto* approach and present examples of the most common and useful applications for in vivo mutagenesis to generate base substitutions, deletions, insertions, as well as for precise in vivo assembly and integration of multiple genetic elements, or gene collage.

Keywords

DNA modification; DNA oligonucleotides; site-directed mutagenesis; gene targeting; *delitto perfetto* system double-strand break; yeast *Saccharomyces cerevisiae*; gene collage

1. Introduction

The yeast *Saccharomyces cerevisiae* is the most well-characterized eukaryotic organism as it has been long utilized for brewing and baking as well as being very easy to grow and manipulate in the laboratory (¹). *Saccharomyces cerevisiae* was the first eukaryote to have the complete genome sequenced (²), and the genome sequencing project led to the discovery of many new yeast genes with unknown function (^{3, 4}). Moreover, as an “honorary mammal,” *S. cerevisiae* has a large number of genes that are homologs of mammalian and human genes (⁵). Thus, functional analysis studies in the yeast model organism shed light on the roles of the corresponding genes in humans and in many other higher eukaryotes. Beyond the simplest experiments of gene disruption or gene knockout, where the original sequence of a gene is replaced with that of a genetic marker (⁶), site-specific mutagenesis of the genes of interest is the most powerful approach of reverse genetics to reveal what phenotypes arise as a result of the presence of particular genes and to generate novel variants of the genes. Thus, the possibility to generate specific point mutations or localized random changes at will, directly in vivo in the DNA locus of choice without leaving behind any marker or other heterologous DNA sequence, provides the opportunity to better understand and modify the role of a given genetic element, or the structure and function of a particular protein. Without leaving any trace, as in the “perfect murder,” the *delitto perfetto* (Italian for

perfect murder) approach to in vivo mutagenesis utilizes simple, precise, and highly efficient tools for engineering the genome of yeast cells with the desired modifications (^{7, 8}). Exploiting the tremendous capacity of *S. cerevisiae* to perform efficient homologous recombination even when very short regions of homology are involved (30–50 bp) (⁶), synthetic oligonucleotides represent the most versatile and high-throughput device for genome engineering in a homology-driven manner (⁸). Moreover, taking advantage of the fact that a double-strand break (DSB) stimulates homologous recombination 1,000–10,000-fold, using the break-mediated *delitto perfetto* system, it is possible to simultaneously generate multiple different mutants or perform more sophisticated genetic rearrangements that would otherwise be too rare to be detected (^{9–11}).

The first step of *delitto perfetto* involves the insertion of a COunterselectable REporter (CORE) cassette containing two markers. Prior to initiating this step, the researcher must decide which CORE cassette to use, taking into account the background of the strain (*See Note 1*) to be mutagenized, the markers currently present within this strain, and the kind of mutation(s) desired. Seven CORE plasmids have been created (Fig. 11.1), including those for a non-break system and a break system, thereby providing the researcher various choices to utilize this technique. Amplification of the chosen CORE cassette from its respective plasmid by polymerase chain reaction (PCR) is accomplished using primers which also contain 50-nucleotide (nt) tails of homology to either side of the target site (Table 11.1 and Fig. 11.2a) to drive the integration of the CORE to its desired location (Fig. 11.2b) in the first step of *delitto perfetto*. The second step involves replacement of the entire cassette with oligonucleotides or larger pieces of DNA to yield the expected modification to the original segment of chromosomal DNA (Fig. 11.2c). The generation of a DSB next to the CORE in the break system enhances the efficiency of targeting more than 1,000-fold (^{9–11}), expanding the applications of the mutagenesis system. From beginning to end, *delitto perfetto* yields the final strain in less than 2 weeks and has proven to be a very useful tool in molecular biology. Examples provided in this review illustrate many changes that can be created through removal of the CORE, such as point mutations, random mutations, deletions, insertions ranging from a few nucleotides to fragments several kilobases in size, and in vivo gene collage.

2. Materials

2.1. Amplification of CORE

1. Seven CORE plasmids are available (*see* Fig. 11.1).
2. DNA primers (Invitrogen, Carlsbad, CA or Alpha DNA, Montreal, Quebec, Canada), desalted and non-purified: 50 pmol/μl. Store at –20°C.
3. Ex Taq DNA polymerase, 10× buffer, 2.5 mM dNTPs (Clontech, Mountain View, CA).

2.2. Gel Electrophoresis

1. Agarose (Fisher, Pittsburgh, PA).
2. TBE running buffer (10×) (Fisher).

3. Prestained molecular weight marker (New England Biolabs, Ipswich, MA).
4. Loading dye (Fisher).

2.3. PCR Product Concentration

1. Ethanol (EtOH): 95 and 70% concentrations.
2. Sodium acetate (NaOAc; Sigma, St. Louis, MO): 3 M (pH 5.2) stock solution, filter sterilized. Store at room temperature (*see Note 2*).

2.4. Transformation Reagents and Media

1. YPD (per 1 l): 10 g yeast extract, 20 g soy peptone, 20 g dextrose (Difco/BD, Franklin Lakes, NJ). For solid media, add 20 g agar (Difco/BD) (*see Note 3*).
2. YPLac liquid (per 1 l): 10 g yeast extract, 20 g soy peptone, 27 ml lactic acid (Difco/BD), pH adjusted to 5.5 with lactic acid (Fisher).
3. Stock solution of 20% high-pure galactose (Sigma) is filter sterilized and stored at room temperature.
4. Lithium acetate (LiOAc; Sigma): Stock of 1 M concentration. Filter sterilize. Store at room temperature.
5. TE 10× stock solution: 100 mM Tris (Fisher) (pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA; Sigma) (pH 7.5). Filter sterilize. Store at room temperature.
6. Polyethylene glycol 4000 (PEG 4000; Sigma): 50% stock solution. Store at room temperature (*see Note 4*).
7. Working solutions: Solution 1 (0.1 M LiOAc, TE 1×, pH 7.5) and solution 2 (0.1 M LiOAc, TE 1×, pH 7.5 in 50% PEG 4000).
8. Solution of salmon sperm DNA (SSD, Roche, Basel, Switzerland), 100 µg/ml. Store at −20°C.
9. SC-Ura (synthetic complete media lacking uracil) solid media (Fisher).
10. Glass beads, approx. 5 mm diameter (Fisher).
11. 5-Fluoroorotic acid (5-FOA; per 1 l): Solution of 5-FOA is prepared by dissolving 1 g 5-FOA (US Biological, Swampscott, MA) in 300 ml of water prior to filter sterilization. 700 ml SD-complete (synthetic dextrose-complete) agar media is autoclaved, then cooled to 55–60°C, and the filtered solution of 5-FOA is then mixed with media prior to pouring.
12. G418 (per 1 l): YPD agar media is autoclaved, then cooled to 55–60°C, and G418 solution (200 µg/ml; US Biological) is then mixed with media prior to pouring. Stock solution is prepared in 50 mg/ml filter-sterilized aliquots and stored at 4°C.
13. Hygromycin B (Hygro; per 1 l): YPD agar media is autoclaved, then cooled to 55–60°C, and Hygro solution (300 µg/ml; Invitrogen) is then mixed with media prior to pouring.

14. YPG (per 1 l): 10 g yeast extract, 20 g soy peptone, 30 ml glycerol (Difco/BD), 20 g agar.
15. Sterile velveteens (Fisher).

2.5. Genotypic Testing of Transformants

1. Lyticase (Sigma) is dissolved at 2,000 U/ml and stored in 1 ml aliquots at -20°C .
2. Taq DNA polymerase, 10 $\times$ buffer, 10 mM dNTPs (Roche).

2.6. Design of DNA Oligonucleotides for Removal of CORE and Generation of Mutations

1. DNA oligonucleotides (Invitrogen or Alpha DNA): 50–100mers, desalted and non-purified (50 pmol/ μl). Store at -20°C .

3. Methods

Despite the efficiency of recombination when a DSB is induced, induction of a DSB may not be required depending on the strain being mutagenized and the type of modification. The DSB system is preferred when multiple mutations are desired simultaneously; when the modification involves gross deletions, insertions, gene fusions, or other genomic rearrangements (¹¹); and when the strain is deficient in homologous recombination functions (¹⁰).

Several combinations of two markers can be used for the *delitto perfetto* technique and are contained within the various CORE cassettes on plasmids (Fig. 11.1). The two CORE markers are used for selection purposes and consist of the following: an antibiotic resistance marker (REporter) – which confers resistance to the antibiotics hygromycin B or Geneticin (G418) – and a COUNTERselectable marker, either the *KIURA3* gene (a *URA3* homolog from *Kluyveromyces lactis*), which can be selected against using 5-FOA, or a marker coding for the human p53 mutant V122A, which is toxic to yeast when overexpressed and can be selected against using a galactose-containing media. In addition, the break system cassettes include the inducible *GAL1* promoter and the gene, used to induce the DSB at the 18-nt I-*SceI* break site.

In the first step of *delitto perfetto*, the CORE is amplified through PCR to attach the tails of homology to the desired chromosomal locus (Fig. 11.2a) and its PCR product is inserted into the cells by transformation (Fig. 11.2b). The CORE cassette will then integrate at the desired genomic locus in approx. $1/10^6$ yeast cells via homologous recombination. Following transformation, the transformant colonies are isolated to observe for insertion of the CORE through phenotypic and genotypic testing. The second step of this technique is a transformation using oligonucleotides or other DNA to remove the entire CORE cassette and introduce the desired mutation(s) (11.2c). See Section 3.6 for details on oligonucleotide design to remove the CORE.

3.1. Amplification of CORE from Plasmid

1. DNA primers will first be used to amplify the CORE from the chosen plasmid. These primers range from 70 to 100 nt in length with an overlap of at least 50 nt

with the genomic targeting region and an overlap of 20 nt with the CORE cassette sequence (Table 11.1). Additionally, in the break-induced system, the 18-nt recognition sequence for the *I-SceI* endonuclease is included in one of the two primers (Table 11.1).

2. PCR conditions: Amplification of the CORE cassette from circular plasmid (about 50 ng) using 50 pmol/ μ l of each primer is performed with high yield in a final volume of 40 μ l using Ex Taq DNA polymerase with a 2 min cycle at 94°C; 32 cycles of 30 s at 94°C, 30 s at 57°C, and 4 min at 72°C (or 5 min at 72°C for cassettes over 4 kb in size); a final extension time of 7 min at 72°C; and samples are held at 4°C. Ex Taq DNA polymerase consistently produces a higher yield of CORE cassette amplification than does Taq DNA polymerase. dNTPs (10 mM) are used for this reaction. An extension time of 1 min/kb is assumed for this reaction.
3. Following PCR, the samples are ready for gel electrophoresis and PCR product concentration.

3.2. Gel Electrophoresis

1. We use a dilution of 0.5 $\times$ TBE running buffer, which is obtained from 10 $\times$ TBE by mixing 50 ml of 10 $\times$ TBE buffer with 950 ml deionized water prior to use.
2. A small aliquot (about 2 μ l) of PCR product is run on a 0.8% agarose gel to observe anticipated band.

3.3. PCR Product Concentration

1. The product of six reactions of PCR is combined for precipitation with a 2.5 $\times$ volume of 95% EtOH and 1/10 3 M NaOAc (pH 5.2) in a microcentrifuge tube. Centrifugation is carried out at maximum speed for 10 min. A small pellet should be visible on the bottom of the tube.
2. The supernatant is discarded and the pellet is washed with 100 μ l of 70% EtOH, paying attention not to detach the pellet. If the pellet is detached, it is necessary to spin again for 5 min and then discard the supernatant. Then, as much EtOH as possible is removed without detaching the pellet.
3. The pellet is then dried in a speed vac for about 15 min and resuspended in 50 μ l of water. Five to 10 μ l are used for each transformation.

3.4. Step 1: Transformation to Insert the CORE

The following transformation protocol is used to first insert the CORE PCR product into the strain of choice and then to drive replacement of the CORE with DNA oligonucleotides or other segments of DNA. This transformation procedure has been modified from the lithium acetate protocol described by Wach et al. (⁶). During the transformation, the LiOAc acts to make the cell wall permeable. The presence of PEG 4000 is used to adhere the DNA to the cells such that the proximity allows for entry into the cells. When transforming to insert the CORE PCR product, SSD is used as carrier DNA and serves as a buffer between the targeting DNA from the PCR and any DNA degradation factors present within the cell. In

the second transformation using oligonucleotides to remove the CORE, the use of SSD is unnecessary as the oligonucleotides at the concentration of 1 nmol/20 µl act as carrier DNA themselves:

1. Inoculate 5 ml of YPD liquid medium with chosen strain and shake at 30°C overnight (O/N) (*see Note 5*).
2. Inoculate 50 ml of YPD liquid medium with 1.5 ml of the O/N culture in a 250-ml glass flask and shake vigorously at 30°C for 3 h.
3. Solutions 1 and 2 are prepared immediately prior to transformation.
4. Transfer culture to a 50-ml conical tube and spin at 1,562×*g* for 2 min.
5. Remove the supernatant and wash cells with 50 ml of sterile water and spin as stated previously.
6. Remove the supernatant and resuspend cells in 5 ml of solution 1 and spin as stated previously.
7. Remove the supernatant and resuspend cells in 250 µl of solution 1. This amount of cells is sufficient for approx. 7–8 transformations.
8. Aliquot 50 µl of the cell suspension in microcentrifuge tubes and add 5–10 µl of concentrated CORE PCR product and 5 µl of SSD (heat-denatured for 5 min at 100°C prior to use and immediately kept on ice), then gently mix by tapping the tube.
9. Add 300 µl of solution 2 for each transformation reaction. Mix briefly by vortexing.
10. Incubate transformation reactions at 30°C for 30 min with shaking.
11. Heat shock at 42°C for 15 min to drive the DNA into the cells.
12. Collect cells by centrifugation at 2,236×*g* for 4 min.
13. Remove the supernatant and resuspend cells well in 100 µl of water.
14. Plate all cells from each transformation tube on one SC-Ura plate using approx. 8–12 sterile glass beads and incubate at 30°C for 2–3 days (*see Note 6*).
15. Using sterile velveteen, replica-plate from SC-Ura to G418- or Hygro-containing media (depending on the CORE used) and incubate at 30°C O/N.
16. Once transformants are observed (typically 5–30 colonies per plate), streak for single-colony isolates on YPD solid media. Incubate at 30°C for 2 days.
17. Make patches of the single colonies on new YPD solid media, along with the original strain, and incubate at 30°C O/N.
18. Replica-plate the grown patches to YPD, SC-Ura, G418, Hygro, YPG to select against petite cells, and any other various selective media depending on the background of your strain, and incubate at 30°C O/N.
19. Following observation of correct phenotype, the samples are ready for genotypic testing.

3.5. Colony PCR of Transformants

1. Resuspend cells (approx. 1 mm^3) in $50 \mu\text{l}$ water containing 1 U of lyticase. Incubate at room temperature for 10 min, followed by incubation in a heat block at 100°C for 5 min.
2. PCR conditions: Colony PCR of the transformant patches presenting the expected phenotypes using $10 \mu\text{l}$ of the cell resuspension solution is accomplished with 50 pmol of each primer, with an expected amplification region between 300 bp and 1.2 kb (see Fig. 11.3). dNTPs (10 mM) are used for this reaction. PCR is performed in a final volume of $50 \mu\text{l}$ using Taq DNA polymerase (Roche) with a 2 min cycle at 95°C ; 32 cycles of 30 s at 95°C , 30 s at 55°C , and 1 min at 72°C ; a final extension time of 7 min at 72°C ; and samples are held at 4°C . An extension time of 1 min/kb is assumed for this reaction (see Note 7).
3. Following PCR, samples are run on a 1% agarose gel (See Section 3.2) for observation of PCR product.
4. Strains are now ready for step 2 to remove the CORE.

3.6. Design of DNA Oligonucleotides for Removal of CORE and Generation of Mutations

Numerous mutations can be accomplished through the use of the *delitto perfetto* technique. These include substitutions, insertions, the generation of random mutations through the use of degenerate oligonucleotides, and deletions. Figure 11.4 illustrates the sequence of oligonucleotides (A–D) needed to produce all of these mutations at the genomic locus indicated in the figure. When substitutions or insertion mutations are desired, the location of the CORE insertion should be next to the region of modification. Conversely, the CORE should replace the entire targeted region when a small deletion or a random mutation is desired. If a large deletion is desired, a CORE with the break system is inserted within the region to be removed (¹¹).

To remove the CORE and generate the mutation with DNA oligonucleotides, the following considerations should be made. The use of a single oligonucleotide is sufficient; however, a pair of complementary oligonucleotides increases the frequency of integration 5–10-fold (¹¹). Additionally, while shorter oligonucleotides (≈ 40 nt) can be used to effectively transform the strain, longer oligonucleotides approaching lengths of 80 nt are more favorable as they increase the efficiency of targeting as well as the window of mutagenesis (¹¹). The external 30–40 nt of the oligonucleotide or oligonucleotide pair are used for efficient homologous recombination to introduce the desired mutation and allow for loss of the CORE. It is of note that once the CORE cassette has been integrated in a specific chromosomal locus, many gene variants can be generated by transforming the cells with oligonucleotides designed to produce different alterations.

3.7. Step 2: Transformation Using DNA Oligonucleotides in Non-break System

1. Inoculate 5 ml of YPD liquid medium with chosen strain and shake at 30°C (O/N).
2. Inoculate 50 ml of YPD liquid medium with 1.5 ml of the O/N culture in a 250-ml glass flask and shake vigorously at 30°C for 3 h.

3. Solutions 1 and 2 are prepared immediately prior to transformation.
4. Transfer culture to a 50-ml conical tube and spin at $1,562\times g$ for 2 min.
5. Remove the supernatant and wash cells with 50 ml of sterile water and spin as stated previously.
6. Remove the supernatant and resuspend cells in 5 ml of solution 1 and spin as stated previously.
7. Remove the supernatant and resuspend cells in 250 μ l of solution 1. This amount of cells is sufficient for approx. 7–8 transformations.
8. Aliquot 50 μ l of the cell suspension in microcentrifuge tubes and add 1 nmole of DNA oligonucleotides (heat-denatured for 2 min at 100°C , then immediately kept on ice prior to use). When using a single oligonucleotide, a 20 μ l volume (at 50 pmol/ μ l) is used or when using a complementary DNA oligonucleotide pair, 10 μ l of each is used. Gently mix the tube by tapping.
9. Add 300 μ l of solution 2 for each transformation reaction. Mix briefly by vortexing.
10. Incubate transformation reactions at 30°C for 30 min with shaking.
11. Heat shock at 42°C for 15 min to drive the DNA into the cells.
12. Collect cells by centrifugation at $2,236\times g$ for 4 min.
13. Remove the supernatant and resuspend cells well in 100 μ l of water.
14. Plate cells from each transformation tube on one YPD solid plate using approx. 8–12 sterile glass beads and incubate at 30°C O/N.
15. Using sterile velveteen, replica-plate from YPD to 5-FOA and incubate at 30°C for 2 days. If necessary, replica-plate again on 5-FOA media to allow for growth of Ura^{-} colonies clearly distinct from the background (*see Note 8*).
16. Using sterile velveteen, replica-plate from 5-FOA to YPD and G418- or Hygro-containing media (depending on the CORE used) and incubate at 30°C O/N.
17. Mark G418-sensitive or Hygro-sensitive colonies on the YPD media and streak for single colonies on new YPD solid media. Incubate at 30°C for 2 days.
18. Make patches of the single colonies on new YPD solid media, along with the original strain, and incubate at 30°C O/N.
19. Replica-plate patches to YPD; SC-Ura; G418; Hygro; YPG, which selects against cells with defective mtDNA; and any other various selective media depending on the background of your strain and incubate at 30°C O/N.
20. Following observation of correct phenotype, the samples are ready for genotypic testing (*see Section 3.5*).
21. PCR samples containing the mutagenized region are now ready for DNA purification and sequencing analysis (*see Note 9*).

3.8. Step 2: Transformation Using DNA Oligonucleotides in Break System

1. Inoculate 50 ml of YPLac liquid medium with chosen strain in a 250-ml glass flask and shake at 30°C (O/N) (*see Note 10*).
2. Add 5 ml of galactose from a 20% solution into the O/N culture to obtain a 2% galactose solution and shake vigorously at 30°C for 3–6 h (*see Note 11*).
3. Solutions 1 and 2 are prepared immediately prior to transformation.
4. Transfer culture to a 50-ml conical tube and spin at $1,562\times g$ for 2 min.
5. Remove the supernatant and wash cells with 50 ml of sterile water and spin as stated previously.
6. Remove the supernatant and resuspend cells in 5 ml of solution 1 and spin as stated previously.
7. Remove the supernatant and resuspend cells in 250 μ l of solution 1. This amount of cells is sufficient for approx. 7–8 transformations.
8. Aliquot 50 μ l of the cell suspension in microcentrifuge tubes and add 1 nmole of DNA oligonucleotides (heat-denatured for 2 min at 100°C, then immediately kept on ice prior to use). When using a single oligonucleotide, a 20 μ l volume (at 50 pmol/ μ l) is used or when using a complementary DNA oligonucleotide pair, 10 μ l of each is used. Gently mix the tube by tapping.
9. Add 300 μ l of solution 2 for each transformation reaction. Mix briefly by vortexing.
10. Incubate transformation reactions at 30°C for 30 min with shaking.
11. Heat shock at 42°C for 15 min to drive the DNA into the cells.
12. Collect cells by centrifugation at $2,236\times g$ for 4 min.
13. Remove the supernatant and resuspend cells well in 100 μ l of water.
14. Plate cells from each transformation tube on one YPD solid plate using approx. 8–12 sterile glass beads and incubate at 30°C O/N. Dilutions may be necessary prior to plating due to the efficiency of oligonucleotide recombination following DSB induction.
15. Using sterile velveteen, replica-plate from YPD to 5-FOA and incubate at 30°C for 2 days. If necessary, replica-plate again on 5-FOA media to allow for growth of Ura⁻ colonies clearly distinct from the background (*see Note 8*).
16. Using sterile velveteen, replica-plate from 5-FOA to YPD and G418- or Hygro-containing media (depending on the CORE used) and incubate at 30°C O/N.
17. Mark G418-sensitive or Hygro-sensitive colonies on the YPD media and streak for single colonies on new YPD solid media. Incubate at 30°C for 2 days.
18. Make patches of the single colonies on new YPD solid media, along with the original strain, and incubate at 30°C O/N.

19. Replica-plate patches to YPD; SC-Ura; G418; Hygro; YPG, which selects against cells with defective mtDNA; and any other various selective media depending on the background of your strain and incubate at 30°C O/N.
20. Following observation of correct phenotype, the samples are ready for genotypic testing (*see Section 3.5*).
21. PCR samples containing the mutagenized region are now ready for DNA purification and sequencing analysis (*see Note 9*).

3.9. The Delitto Perfetto Approach to Insert a Large DNA Fragment

In Fig. 11.5, the two-step process shown illustrates the insertion of a large segment of DNA, 10 kb in size. Generally, an insert of these proportions is obtained through amplification of the sequence through PCR, which, although possible, greatly increases the risk of introducing several mutations through the extension process. In our system, the large DNA of interest is carried on a plasmid which is linearized prior to transformation. Linearization of the plasmid is required to generate free DNA ends and stimulate homologous recombination. The large segment of plasmid DNA is integrated into genomic DNA at a chosen location by in vivo recombination following co-transformation of the linearized plasmid carrying the fragment and two pairs of complementary oligonucleotides. Each pair contains regions of homology on either side of the target site in addition to homology with the 10-kb fragment, thereby directly driving it into its desired locus. This way, sequencing analysis is not required following integration of the large fragment.

3.10. The Delitto Perfetto Approach to Insert Multiple Sequences for Gene Collage

It is also possible to insert two or more sequences or genes next to each other simultaneously using *delitto perfetto*, as seen in Fig. 11.6. To accomplish this, the genes or segments of interest are amplified in such a way that the primers of each PCR fragment have tails of homology to the sequence of the contiguous segment and the most external primers contain homology to the target site. Through co-transformation with these multiple PCR products, the individual pieces recombine in vivo as a form of gene collage, while the outlying primers drive integration into the genome at the desired locus.

4. Notes

1. This review focuses on the generation of engineered haploid strains of yeast. For the use of *delitto perfetto* in diploid cells, refer to ⁽¹¹⁾ for a detailed explanation of modifications to the protocol.
2. For media preparation, deionized water is used. All other uses of the term “water” in this chapter, however, refer to deionized water that was sterilized by filtration or autoclaving.
3. Unless otherwise noted, all solid media are to be stored at 4°C. Exceptions include YPD liquid and agar, which we store at room temperature.

4. PEG 4000 solution will be extremely viscous, so filter sterilizing can take up to 1 h depending on the volume. Autoclaving is an alternative means of sterilization for this solution.
5. Using 50-ml conical tubes for O/N growth is preferred to using 15-ml tubes, as the larger size allows for greater dispersion of the nutrients in the broth to each of the cells. Additionally, *S. cerevisiae* is an aerobic species, so lids should not be capped tightly but instead loosely cover the tube and secured with tape.
6. When inserting CORE, if growth on SC-Ura media is not observed after 3 days (*see Note 8* below), the transformation can be performed by plating onto YPD and incubating at 30°C O/N followed by replica-plating to G418- or Hygro-containing media, depending on the CORE used, and incubating at 30°C for 2–3 days until large colonies appear. This would then be followed by replica-plating to SC-Ura and incubating at 30°C O/N.
7. As little as 5 µl of the cell resuspension solution can be used per reaction; however, this is not optimal when sequencing is necessary and a volume of 10 µl is suggested for this process.
8. The following is specific to using CORE-UK, CORE-UH, GSKU, and GSHU, as this does not apply to the other cassettes. When *KIURA3* is inserted in the same orientation as the targeted gene, interference from that gene's promoter during transcription may lead to delayed growth on SC-Ura in the first step of *delitto perfetto* (*see Note 6* above) and may increase the number of background cells on 5-FOA in the second step. Depending on insertion orientation of the CORE, a second round of replica-plating to 5-FOA may be needed. Therefore, it is optimal to insert the cassette in such a way that *KIURA3* is oriented opposite to the gene being targeted.
9. Upon successful colony PCR of transformants containing the newly introduced CORE sequence, sequencing analysis is not required. The resulting antibiotic resistance and Ura⁺ phenotype of the strain in addition to the results of the colony PCR are sufficient to provide evidence for successful incorporation of the CORE into the targeted site. Sequencing is, however, necessary to verify the correct insertion of the desired mutation(s). Since the oligonucleotides used are non-purified, the expected additional mutations are in the range of 10–20%. Therefore, it is always better to obtain 3–5 clones for sequencing.
10. YPLac is used to provide a neutral carbon source for the cells prior to addition of galactose. However, cells grow much slower in this medium. It is, therefore, optimal to inoculate cells into YPLac at least 18–20 h prior to the transformation.
11. Addition of galactose activates the inducible *GALI* promoter which regulates the I-*SceI* gene. Experience has shown that longer induction (5–6 h) produces greater efficiency.

Acknowledgments

We thank the members of our lab for their contributions to the editing and revision of this work, notably Rekha Pai, Patrick Ruff, and Ying Shen. We also thank Lee Katz for assistance in proofreading and revision. This work was funded in part by the Georgia Cancer Coalition grant R9028 and the NIH R21EB9228.

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Fig. 11.1.

The CORE plasmids used in the *delitto perfetto* technique. Each of the five plasmids used in the non-break system (**a**) contains a counterselectable marker, either *KIURA3* from *Kluyveromyces lactis* or a mutant form (V122A) of the human p53 cDNA, and a reporter marker, either *kanMX4* conveying resistance to Geneticin (G418) or *hyg* for resistance to the antibiotic hygromycin B. In addition to these markers, the two plasmids used in the break system (**b**) contain the inducible *GAL1* promoter and *I-SceI* gene used to express the *I-SceI* endonuclease and generate a DSB at the *I-SceI* site. The origin of replication (*ori*) for all CORE plasmids is indicated as well as the *bla* marker gene, which provides resistance to the β -lactam antibiotic ampicillin and is used for selection.

Fig. 11.2.

The two-step process of *delitto perfetto*. (a) Step one involves the amplification of a CORE cassette by PCR (portions of primers used for amplification indicated by *thinner line* and *arrow*). (b) The primers create tails of homology to either side of the target region (indicated by *thicker line*) for integration into the genome using the cell's homologous recombination machinery. In this example, the use of the break system CORE cassette GSHU is illustrated. Note that the primer amplifying from the *GAL1-I-SceI* side of the cassette introduces the 18-nt *I-SceI* recognition site (*black box*). This site is utilized in the second step (c) when the *I-SceI* endonuclease expression is turned on with galactose to generate a DSB prior to replacement of the CORE with an oligonucleotide sequence, which introduces the desired mutation. This example uses a single-stranded oligonucleotide to enact this change; however, a pair of complementary oligonucleotides have been shown to increase the efficiency of gene targeting.

SCHEME OF PRIMER PAIRS TO VERIFY CORE INSERTION OR REMOVAL

Fig. 11.3.

Scheme of primer pairs used for colony PCR. Primers should be designed to allow for amplification of the target region in addition to being paired with primers internal to the CORE. The sizes of colony PCR products should range between 300 bp and 1.2 kb. Using this approach, verification of the CORE's integration as well as its replacement can be made. See Table 11.1 for a list of primers and their sequences used to verify the integration of the various CORE cassettes.

Fig. 11.4.

Examples of single oligonucleotide-driven mutations generated using the *delitto perfetto* technique. When a substitution or an insertion mutation is desired (*A, B*), the CORE should be placed next to the target region prior to replacement with a single or complementary oligonucleotide(s). In this example, the original sequence in the genome is provided as a reference at the top of the figure. (*A*) A substitution of a guanine, marked by an *asterisk* above the bolded G on the oligonucleotide, is made in place of the adenine residue on the top strand of the reference sequence (*boxed*). (*B*) An insertion mutation in the original sequence is created through the use of an oligonucleotide containing additional nucleotides (GCGG, marked in *bold*) which are inserted between the adenine and thymine indicated in the reference. When random mutations or small deletions (<5 kb) are desired (*C, D*) in a specific region, it is preferred to delete the region of interest along with the CORE insertion, as the successive targeting event with the oligonucleotides or other DNA will then eliminate the CORE and introduce the desired changes. The region of mutagenesis in the original sequence is bolded and indicated by the *bracket*. (*C*) For the generation of random mutations, the oligonucleotide sequence contains a stretch of 10 bolded Ns, which indicate that any of the four DNA bases can be used when the oligonucleotides are synthesized. The exact degree of this randomness is determined by the investigator. (*D*) The segment of the reference sequence indicated by the *bracket* can be removed through the use of oligonucleotides missing this fragment. In the example, the location of the deleted nucleotides is indicated by the *dashed line*.

Fig. 11.5.

Insertion of a large segment of DNA from a plasmid. In this example, the break system is used to drive the integration of a 10-kb fragment from a plasmid into the target region. **(a)** First, the GSHU CORE cassette and the 18-nt I-SceI break site are inserted into the target region. **(b)** Next, the plasmid carrying the sequence of interest to be integrated within the genome is linearized by restriction digestion outside of the fragment to be inserted. Complementary pairs of oligonucleotides have regions of homology to both the upstream and downstream portions of the sequence of interest to be integrated, as well as to either side of the target region. The linearized plasmid and oligonucleotides are co-transformed into yeast cells following DSB induction at the I-SceI site. By homologous recombination, the large sequence of interest is integrated into the genomic DNA at the specific site without the need for PCR amplification, which otherwise increases the likelihood of unwanted mutations during the polymerization process.

Fig. 11.6.

Mechanism of in vivo gene collage by the *delitto perfetto* approach. (a) In step one, the GSKU CORE cassette is amplified through PCR, with the 18-nt I-SceI break site included within the sequence of one primer. This product is inserted into the target locus. (b) Prior to replacement of the cassette, a preparation step to generate PCR fragments is performed. For this example, a gene of interest will be attached to a chosen promoter and terminator sequences and all components will be inserted at a chosen locus. (c) In step two, the multiple PCR fragments assemble together in vivo by recombination to form a large fragment, which then replaces the GSKU cassette as it integrates into its specific region of the chromosome.

Table 11.1
Primers for CORE Cassette Amplification and Verification of CORE Cassette Insertion

Plasmid ^a	Primers to amplify CORE ^b	Cassette ^c	Markers ^c	Primers for testing cassette insertion ^d
pCORE	P.1 5' -... GAGCTCGTTTTCGACACTGG - 3'	CORE	<i>kanMX4</i>	K2 5' - AGTCGTCACTCATGGTGATT - 3'
	P.2 5' -... TCCTTACCATTAAGTTGATC - 3'	3.2 kb	<i>KlURA3</i>	URA3.2 5' - AGACGACAAAGGCGATGTCAT - 3'
pCORE-UK	P.I 5' -... TTCGTACGCTGCAGGTCGAC - 3'	CORE-UK	<i>KlURA3</i>	URA3.1 5' - TTCAATAGCTCATCAGTCGA - 3'
	P.II 5' -... CCGCGCGTTGGCCGATTTCAT - 3'	3.2 kb	<i>kanMX4</i>	K1 5' - TACAATCGATAGATTGTGCGCAC - 3'
pCORE-UH	P.I 5' -... TTCGTACGCTGCAGGTCGAC - 3'	CORE-UH	<i>KlURA3</i>	URA3.1 5' - TTCAATAGCTCATCAGTCGA - 3'
	P.II 5' -... CCGCGCGTTGGCCGATTTCAT - 3'	3.5 kb	<i>hyg</i>	HI 5' - CCATGGCTCCGCGACCGGCTGC - 3'
pCORE-Kp53	P.I 5' -... TTCGTACGCTGCAGGTCGAC - 3'	CORE-Kp53	<i>kanMX4</i>	K1 5' - TACAATCGATAGATTGTGCGCAC - 3'
	P.II 5' -... CCGCGCGTTGGCCGATTTCAT - 3'	3.7 kb	<i>GALLI/10-p53</i>	p53.2 5' - GACTGTACCACCACCATCCACT - 3'
pCORE-Hp53	P.I 5' -... TTCGTACGCTGCAGGTCGAC - 3'	CORE-Hp53	<i>hyg</i>	HI 5' - CCATGGCTCCGCGACCGGCTGC - 3'
	P.II 5' -... CCGCGCGTTGGCCGATTTCAT - 3'	4.0 kb	<i>GALLI/10-p53</i>	p53.2 5' - GACTGTACCACCACCATCCACT - 3'
pGSKU	P.I 5' -... TTCGTACGCTGCAGGTCGAC - 3'	GSKU	<i>KlURA3 kanMX4</i>	URA3.1 5' - TTCAATAGCTCATCAGTCGA - 3'
	P.IIS 5' -... TAGGGATAACAGGGTAAT CCGCGCGTTGGCCGATTTCAT - 3'	4.6 kb	<i>GALLI-1-SceI</i>	GalE 5' - CTAAGATAATGGGGCTCTTT - 3'
pGSHU	P.I 5' -... TTCGTACGCTGCAGGTCGAC - 3'	GSHU	<i>KlURA3 hyg</i>	URA3.1 5' - TTCAATAGCTCATCAGTCGA - 3'
	P.IIS 5' -... TAGGGATAACAGGGTAAT CCGCGCGTTGGCCGATTTCAT - 3'	4.8 kb	<i>GALLI-1-SceI</i>	GalE 5' - CTAAGATAATGGGGCTCTTT - 3'

^aThere are seven CORE plasmids available.

^b Amplification of the plasmids by PCR can be accomplished by creating primers with the above-listed sequences that are internal to the CORE and an external region homologous to the region in which the CORE will be inserted. The primers used to amplify the cassettes from pGSKU and pGSHU require the addition of the 18-nt I-SceI recognition site (**bold**) next to the *GAL1* promoter.

^cThe sizes and composition of the cassettes vary depending on the markers present.

^dPrimers and their sequences used for verification of CORE integration and replacement are provided.