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Single-Step Gene Knockout of the *SUC2* Gene in *Saccharomyces cerevisiae*: A Laboratory Exercise for Undergraduate Students[†]

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INTRODUCTION

Gene knockout technology is widely used to study the function of a gene product. The generation of a knockout strain of a specific organism and the comparison of its phenotype with that of the parental strain helps students understand the concept of information flow in biological systems (i.e., from DNA to RNA to protein). Historically, gene knockouts have been mostly generated from bacteria and yeast as these organisms are particularly efficient in homologous recombination (1, 2). The yeast *Saccharomyces cerevisiae* can multiply as a haploid or a diploid. The use of haploids greatly simplifies the construction of knockouts as only a single copy of the gene of interest needs to be targeted (3).

The *SUC2* gene encodes the enzyme invertase, which converts the disaccharide sucrose into glucose and fructose (4). Invertase is secreted by yeast cells. It is also frequently used as a model for the study of enzyme kinetics in laboratory teaching courses (5).

This article describes a procedure to knock out the *SUC2* gene in *S. cerevisiae* by a single-step PCR knockout method followed by confirmation of the gene deletion at the phenotypic level by measuring invertase activity. The project is achievable in the teaching laboratory and supports laboratory skills, bioinformatics skills, and scientific thinking. A brief overview of the whole experiment is shown in Figure 1.

PROCEDURE

General overview

A schematic representation of the knockout strategy can be found in Figure 2B. Briefly, a PCR-mediated single-step

gene deletion method is used (6, 7). In this course, plasmid pHIPH4 (available at Addgene [<https://www.addgene.org>], a nonprofit plasmid repository) is used as a template (Fig. 2A). This plasmid contains the ampicillin resistance marker for selection in *E. coli* as well as the hygromycin B resistance gene for selection in yeast. Expression of the marker in yeast is regulated by the *TEF1* promoter and terminator in yeast and by the *EM7* promoter in *E. coli* (8). The forward primer (5'-ATGCTTTTGAAGCTTTCCTTTTCCTTTGGCTG GTTTTG-CCCACACACCATAGCTTCAA-3') contains 20 nucleotides (nt) corresponding to the yeast *TEF1* promoter of plasmid pHIPH4 at the 3'-end and 40 nt corresponding to the start of the *SUC2* open reading frame (ORF) at the 5'-end. The reverse primer (5'-CTATTTTACTTCCCT TACTTGGAACTTGTAATGTAGAAC-CGTTTTTCGA CACTGGATGGC-3') contains 20 nt corresponding to the transcription terminator present on plasmid pHIPH4 at the 3'-end and 40 nt corresponding to the end of the *SUC2* ORF at the 5'-end. Subsequently, a PCR reaction is performed using plasmid pHIPH4 as a template. The resulting PCR product contains a hygromycin B resistance gene cassette including promoter, hygromycin B coding sequence, transcriptional termination sequences and the 40 bp flanking regions that are necessary for homologous recombination in the *S. cerevisiae* genome at the *SUC2* locus (Fig. 2B). After transformation in competent yeast cells, colonies are selected for hygromycin B resistance. Integration at the correct locus is verified by PCR using chromosomal DNA of the transformed yeast strain as a template. Knockout of the gene is confirmed using the invertase assay on the resulting transformants.

PCR of the knockout cassette

The inexpensive *Taq* polymerase enzyme can be used to perform PCR. There is no need to purify the PCR products. An example of the PCR result can be found in Figure 3A.

Transformation of competent yeast cells

The relatively simple and inexpensive lithium-acetate method is used to make competent yeast cells (9). It is

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important to use a *S. cerevisiae* strain that is defective in mating type switching. A period of two days of incubation at

30°C is required to obtain colonies large enough to be easily picked by students for subsequent culturing. An example of the transformation result can be seen in Figure 3B.

Flowchart

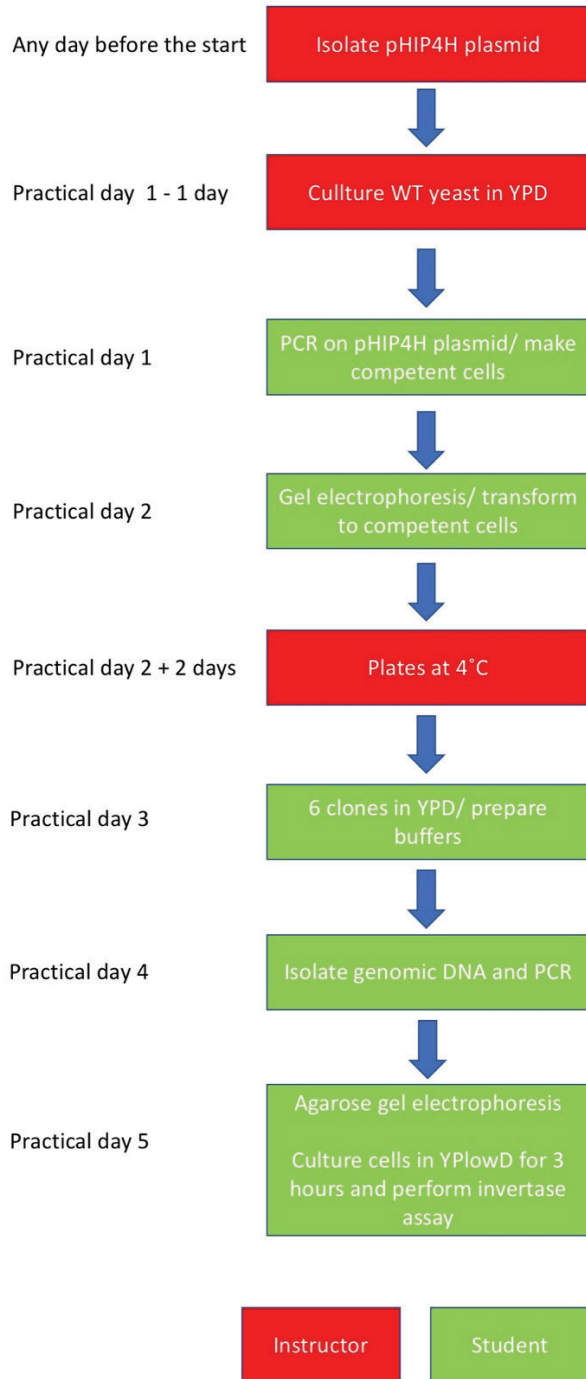


FIGURE 1. Flowchart of the experiment. Instructor activities are shown in red. Student activities are shown in green. Days are related to student activities. For example: “Practical day 1 – 1 day” means that instructors need to start this activity one day prior to the first practical day in which students participate. WT = Wild-Type; YPD = yeast-extract peptone dextrose.

Selection of integration at the genetic level

To verify the integration at the correct locus, PCR is performed after genomic DNA isolation. A forward PCR primer (forward check primer, 5'-GTCCGCAA-CATCTTCTTCTG-3') is designed in the hygromycin B resistance ORF. A reverse PCR primer (reverse check primer, 5'-GCTAAAGCCCTTTAGAATGG-3') is designed downstream of the *SUC2* ORF (Fig. 2B). Using this procedure, we found that one-third (4 out of 12) of the hygromycin B resistant clones contained the cassette at the correct locus. Figure 3C is based on a preselected set of transformants using a screening PCR (data not shown). The actual efficiency of recombination is determined by the size of the flanking

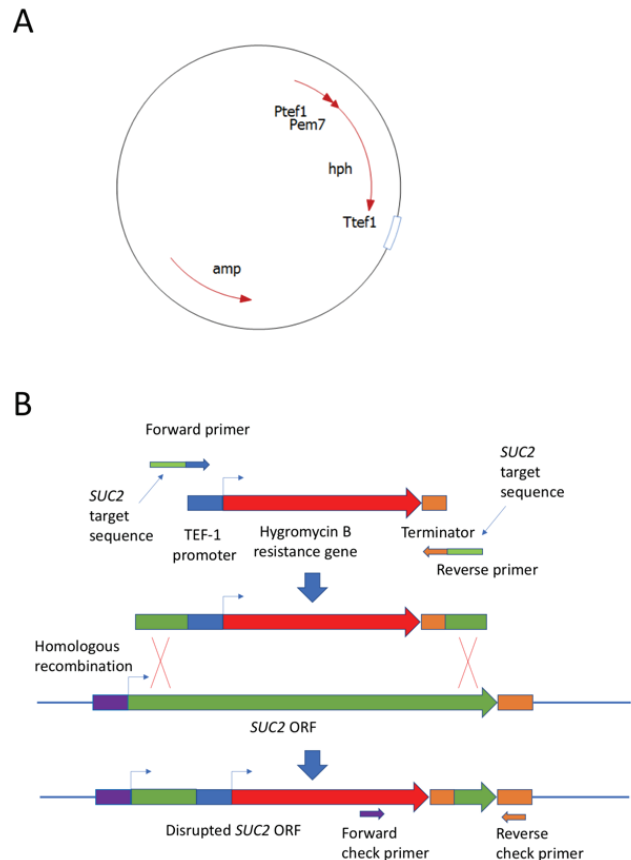


FIGURE 2. Schematic overview of the PCR-mediated single-step gene disruption method. A) Schematic representation of vector pHIP4H containing the hygromycin B gene (hph). B) The oligonucleotides contain flanking sequences corresponding to the *SUC2* gene. PCR is used to amplify a gene knockout cassette harboring a hygromycin B resistance cassette. Upon transformation in yeast, the target gene will be disrupted by two homologous recombination events at the *SUC2* locus. Clones can be selected for resistance toward hygromycin B. ORF = open reading frame.

region. Increasing the size of the flanking region is expected to result in higher efficiencies.

Investigating the phenotype using the invertase assay

Invertase activity can be measured indirectly using 3,5-dinitrosalicylic acid (DNS) reagent. This reagent reacts with reducing sugars to form 3-amino-5-nitrosalicylic acid, which strongly absorbs light at 540 nm. It is important to grow yeast cells in medium containing a low concentration of glucose (0.1% w/v) prior to enzyme measurement, as glucose represses the expression of the *SUC2* gene (10). Invertase is found both intracellular and in a secreted form (11). The enzyme can be obtained in the culture medium, but we found higher activity on cell lysates. After growth of the cells, cells are collected by centrifugation and lysed by freeze-thaw cycles. The resulting lysate is used for the enzyme assay. An example of the invertase assay results is shown in Figure 3D.

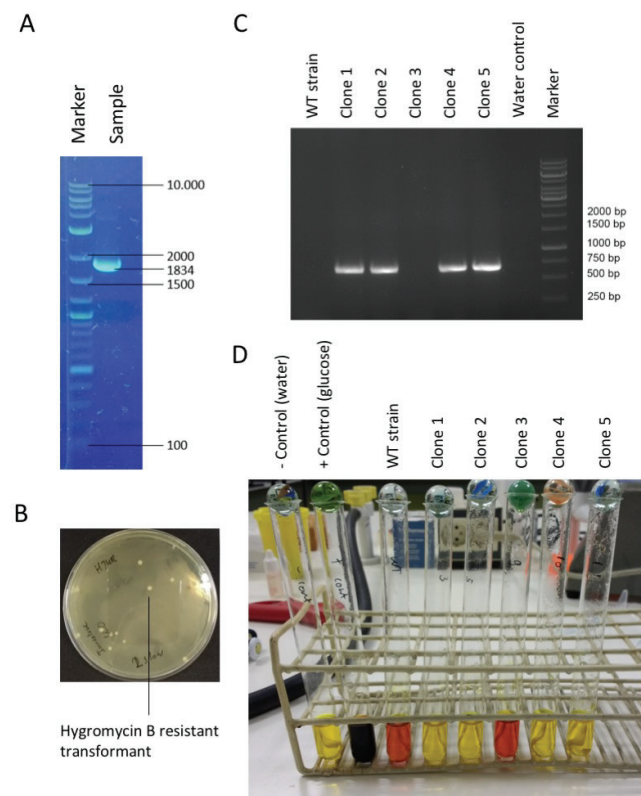


FIGURE 3. Overview of the data that students are expected to generate. A) The knockout cassette PCR product after agarose electrophoresis. Numbers represent base pairs. B) Hygromycin B-resistant yeast colonies. C) A PCR check for the confirmation of integration at the correct locus. This will show a 687 bp band after agarose electrophoresis. Numbers represent base pairs. Based on the PCR results, clones 1, 2, 4, and 5 are considered *SUC2* knockout whereas clone 3 is considered WT. D) Result of the invertase assay. Red color indicates the presence of reducing sugars. Based on the invertase measurements, clones 1, 2, 4, and 5 are considered *SUC2* knockout whereas clone 3 is considered WT. WT = Wild-Type; bp = base pairs.

Safety issues

This experiment involves the use of genetically modified cells and, depending on your country, laws on biosecurity of genetically modified organisms are likely to apply. In the United States, the work with genetically modified cells presented in this article is at the BSL1 level. Instructors and students should be adequately trained to work with genetically modified cells and follow the ASM Biosafety Guidelines (<https://www.asm.org/index.php/guidelines/safety-guidelines>).

DNA staining dyes are used in the procedure. We use Midori green as a safer alternative to ethidium bromide.

CONCLUSIONS

The above described experiment is an easily scalable and relatively inexpensive, yet challenging, research project that couples genetic with biochemical approaches. Last fall, about 90 students attended our course, and the majority of students were successful in generating the knockouts. Tutors can freeze PCR products, competent cells, and knockout clones as failsafes for students. In theory, any non-essential ORF can be targeted using this strategy, so tutors may vary the enzyme to be targeted as well as the enzyme assay to be performed. The invertase assay part may be expanded by classical graphical representation of enzyme kinetics using Lineweaver-Burk plots and Michaelis-Menten kinetics.

SUPPLEMENTAL MATERIALS

- Appendix 1: PDF file of the protocol for students
- Appendix 2: PDF file with instructor recourses

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