

## **A protocol for genome-editing of *Saccharomyces cerevisiae***

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This protocol describes the procedures to perform genome-editing of budding yeast using the genome-editing plasmid backbone vectors published in the following paper. If you have any questions or inquiries, please feel free to send an email to the author.

### **Simple-to-use CRISPR-SpCas9/SaCas9/AsCas12a vector series for genome editing in *Saccharomyces cerevisiae*.**

Satoshi Okada, Goro Doi, Shitomi Nakagawa, Emiko Kusumoto, and Takashi Ito

*G3 Genes/Genomes/Genetics*, jkab304, <https://doi.org/10.1093/g3journal/jkab304>

#### **1) The schedule of genome-editing**

The following summarizes the shortest genome editing schedule (about 2 weeks) when using the genome-editing plasmid backbone vectors created in this project.

- Day 1 Select targets / design and order oligo DNAs
- Day 2 (awaiting the arrival of oligo DNAs)
- Day 3 Receive oligo DNAs / perform Golden Gate Assembly / transform E. coli cells
- Day 4 Inoculate E. coli cells for plasmid extraction / inoculate yeast cells for genome-editing transformation
- Day 5 Extract plasmid from E. coli cells / prepare PCR fragments / transform yeast cells
- Day 6 (awaiting yeast colony formation)
- Day 7 (awaiting yeast colony formation)
- Day 8 (awaiting yeast colony formation)
- Day 9 Pick up yeast colonies
- Day 10 Perform yeast colony PCR / inoculate yeast cells in YPAD liquid
- Day 11 Begin single colony isolation
- Day 12 (awaiting yeast colony formation)
- Day 13 Pick up yeast colonies
- Day 14 Perform yeast colony PCR

## 2) Recommended genome-editing plasmid backbone vectors

We recommend the following three types of vectors, all of which encode the Cas protein and guide RNA (gRNA) scaffold on a single plasmid and use the *GAL1* promoter for Cas protein and gRNA expression. All plasmids are centromeric. The marker for budding yeast is *URA3*, and the marker for *E. coli* is *AmpR*. (Figure 1)

**For SpCas9** (PAM=NGG, the target sequence is located at the 5' side of the gRNA scaffold)

**(16-15) pGAL1-Cas9-tADH1-pGAL1-2Bsal-sgRNAFE(empty)-HDV-tCYC1-CU**

<https://yeast.nig.ac.jp/yeast/by/PlasmidDetail.jsf?id=9747>

**For SaCas9** (PAM=NNGRRT, the target sequence is located at the 5' side of the gRNA scaffold)

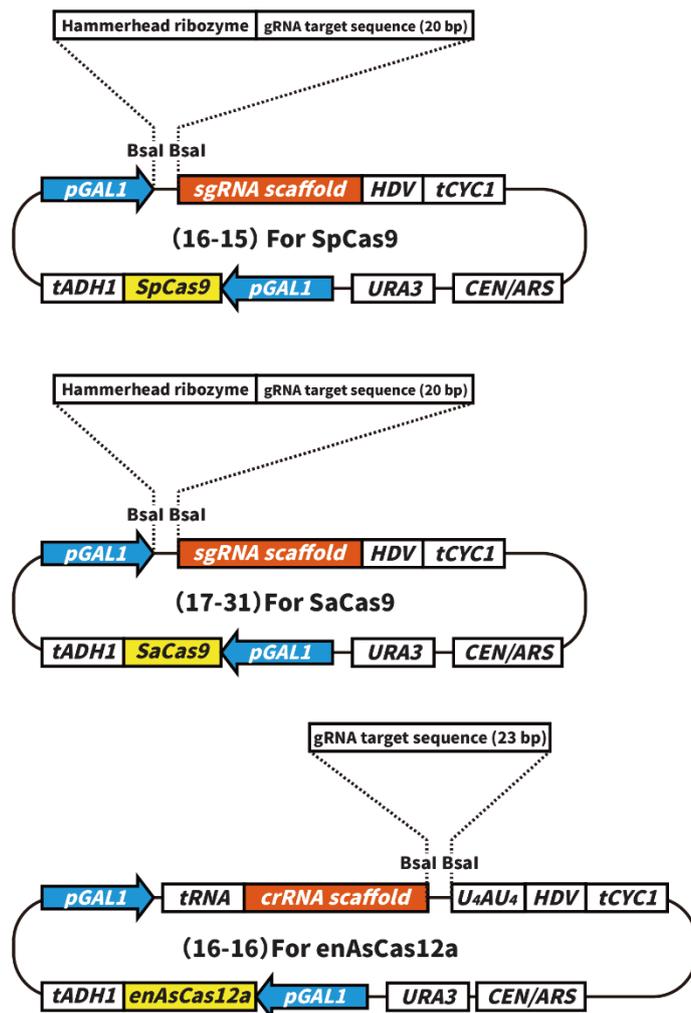
**(17-31) pGAL1-ySaCas9-tADH1-pGAL1-2Bsal-SagRNA(empty)-HDV-tCYC1-CU**

<https://yeast.nig.ac.jp/yeast/by/PlasmidDetail.jsf?id=9748>

**For enAsCas12a** (PAM=TTTV, the target sequence is located at the 3' side of the gRNA scaffold)

**(16-16) pGAL1-yenAsCas12a-tADH1-pGAL1-tRNA-AscrRNA(empty)-2Bsal-U4AU4-HDV-tCYC1-CU**

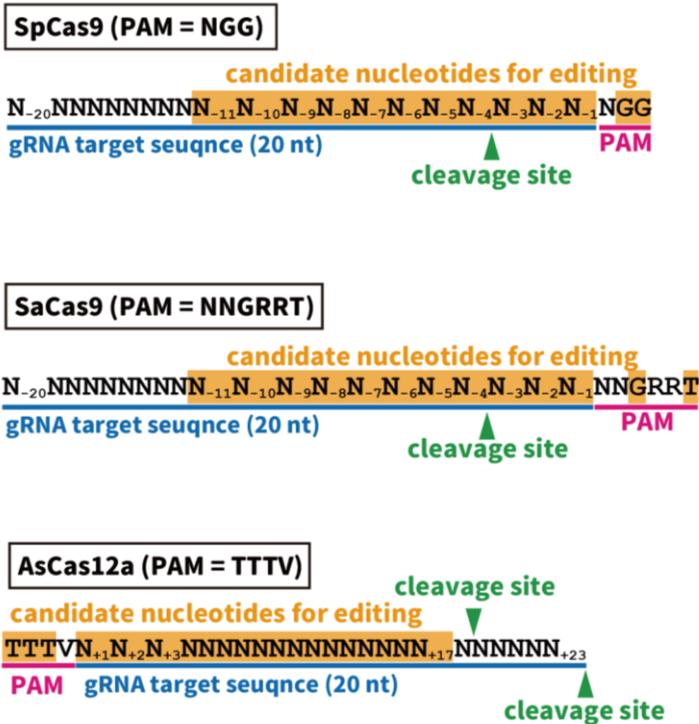
<https://yeast.nig.ac.jp/yeast/by/PlasmidDetail.jsf?id=9750>



(Figure 1) The recommended genome-editing plasmid vectors.

### 3) Select target sequences for gRNAs

When selecting gRNA target sequences, it is essential to understand the positions of the nucleotides that can be edited by each CRISPR/Cas system and to understand which type of genome editing pattern is required for the strain you want to create. The positions of the nucleotides that can be edited by each CRISPR/Cas system are summarized in Figure 2.

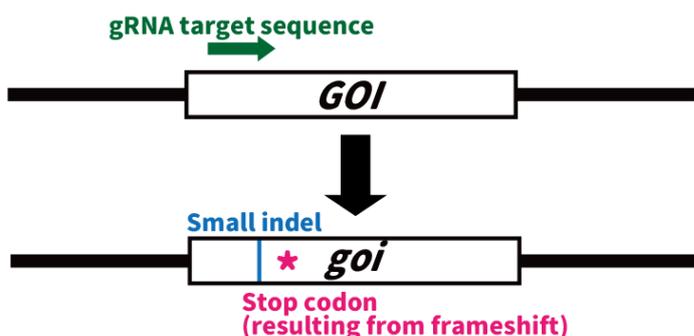


(Figure 2) Locations of gRNA target sequences, PAMs, cleavage sites, and the “editable” nucleotides using SpCas9, SaCas9, and AsCas12a.

Typical genome-editing patterns are summarized below, focusing on what to take care of when selecting gRNA target sequences.

#### 3A) Disruption of gene function by indel (Figure 3)

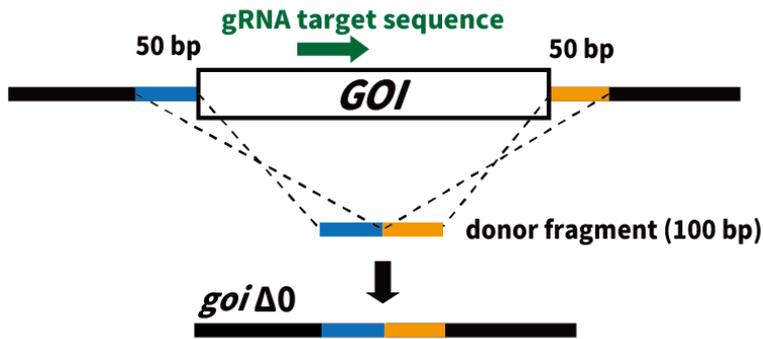
This method expects that indel will cause a frameshift, resulting in a premature appearance leading to the destruction of the gene function. Since the stop codon should appear as early as possible, it is recommended to set the target sequence as close as possible to the 5' side (N-terminal side of the protein) in the ORF of the gene to be disrupted.



(Figure 3) Genome-editing to disrupt gene function by indel.

3B) Deletion of an entire ORF (Figure 4)

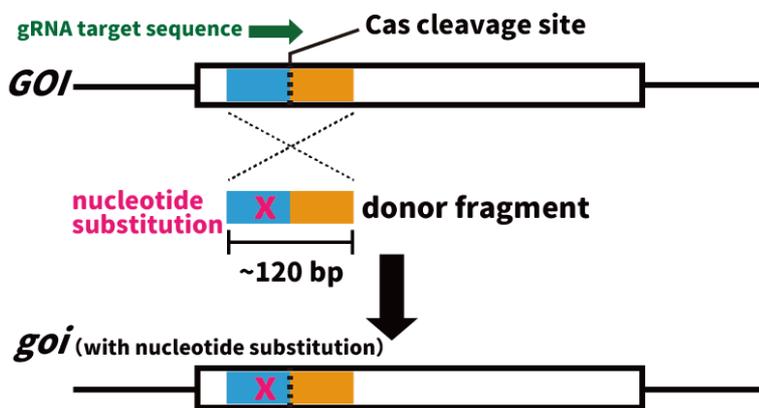
The target sequence can be located anywhere within the ORF to be deleted.



(Figure 4) Genome-editing to delete an entire ORF.

3C) Introduction of nucleotide substitution(s) at specific position(s) (Figure 5)

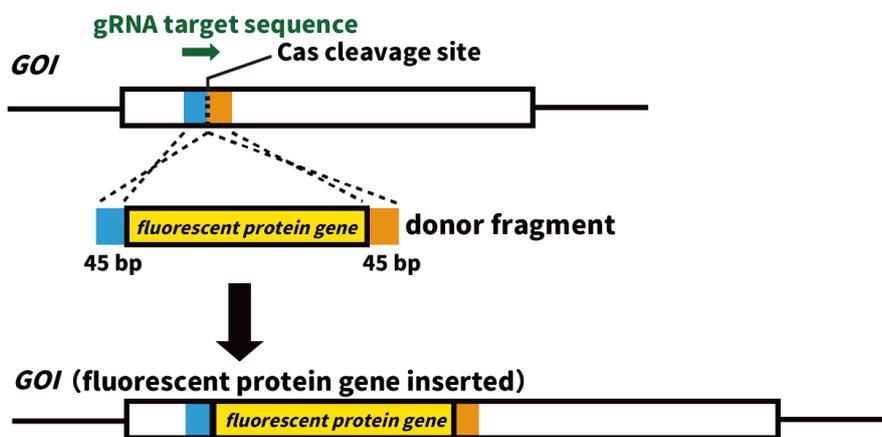
The design should be such that the nucleotide substitution changes one or more nucleotides in the orange area of the target sequence shown in Figure 1.



(Figure 5 ) Genome-editing to introduce a nucleotide substitution at a specific position.

3D) Insertion of a gene fragment (fluorescent protein, tag, etc.) at a specific position (Figure 6)

The insertion of the gene fragment should be designed to cause a change of one or more bases in the orange area of the target sequence shown in Figure 1.



(Figure 6) Genome-editing to insert a gene fragment at a specific position.

In selecting target sequences for gRNAs, it is important to choose sequences with as few off-targets as possible. We use the following two web tools. Both tools show the number of potential off-targets, and we select target sequences with as few potential off-targets as possible. Factors other than the number of potential off-targets (such as GC content and chromatin structure) are not considered when we perform genome editing.

### CRISPRdirect

<https://crispr.dbcls.jp/>

SpCas9 and SaCas9 are supported.

### CRISPOR

<http://crispor.tefor.net/>

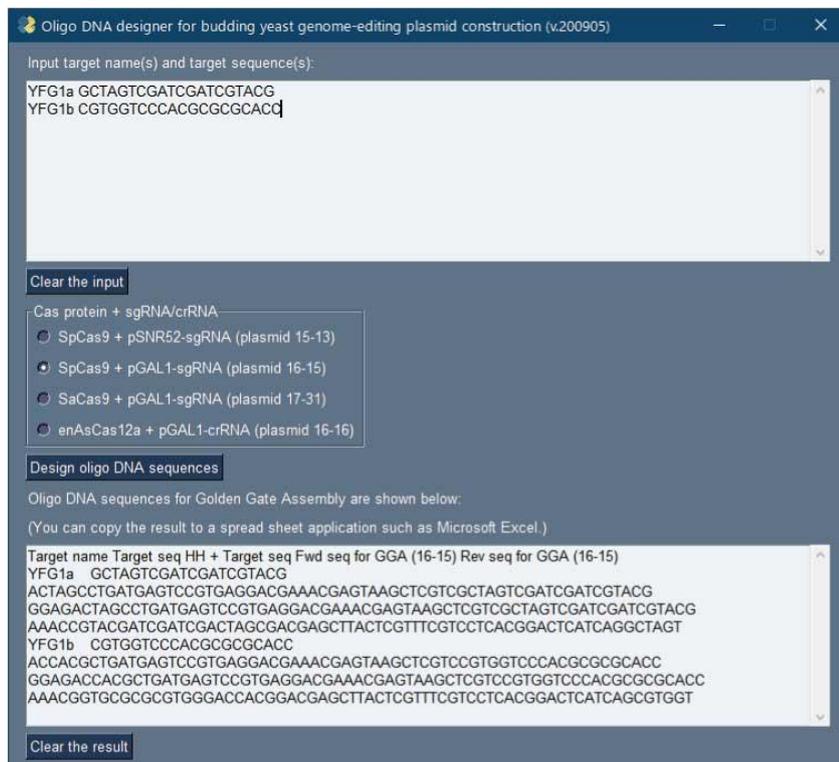
SpCas9, SaCas9, and AsCas12a are supported. The target sequences designed for AsCas12a can be used for enAsCas12a (since the PAM of enAsCas12a includes the PAM of AsCas12a).

## 4) Design oligo DNAs for Golden Gate Assembly

Scripts for oligo DNA design are available in our GitHub repository.

[https://github.com/poccopen/Genome\\_editing\\_plasmid\\_for\\_budding\\_yeast](https://github.com/poccopen/Genome_editing_plasmid_for_budding_yeast)

Enter the names and DNA sequences of the target sequences, select a backbone vector to be used, and the software will automatically calculate the oligo DNA sequences for Golden Gate Assembly according to the target sequences and the backbone vector.



(Figure 7) Screenshot of the program to calculate oligo DNA sequences for Golden Gate Assembly.

## **5) Construct genome-editing plasmids with Golden Gate Assembly**

### 5-1) Preparation of insert fragments for Golden Gate Assembly

Prepare an insert fragment (dsDNA made by hybridizing two oligo DNAs, phosphorylated on the 5' side) for use in Golden Gate Assembly.

Procedures:

[Phosphorylation of oligo DNAs at their ends and hybridization of the phosphorylated oligo DNAs]

Prepare the following reaction solution using a 0.2-mL tube for PCR and mix well by vortexing. It is convenient to prepare a master mix, excluding the oligo DNAs.

H<sub>2</sub>O 13.5 uL

10x T4 DNA ligase buffer 2 uL

Forward oligo DNA (100 uM) 2 uL

Reverse oligo DNA (100 uM) 2 uL

T4 PNK 0.5 uL

Incubate in a thermal cycler. (Program settings are as follows: the lid temperature is set to 100°C.)

37°C 30:00

95°C 10:00

95°C→85°C -2°C /sec °C

85°C→25°C -0.3°C /sec

After the incubation, dilute the double-stranded insert fragment 100-fold with H<sub>2</sub>O. To minimize dilution error, we recommend repeating the 10-fold dilution twice.

### 5-2) Golden Gate Assembly

Procedures:

Join the backbone plasmid and the insert fragment using Golden Gate Assembly.

Reagent:

NEB Golden Gate Assembly Kit (Bsal-HF v2)

<https://www.nebj.jp/products/detail/2097>

Procedures:

Prepare the following reaction mixture using a 0.2-mL tube for PCR and mix well by vortexing.

It is convenient to prepare a master mix without insert fragments.

Backbone plasmid vector 0.5 uL  
Insert fragment (100-fold diluted at 5-1) 0.5 uL  
T4 DNA Ligase Buffer (10x) 1 uL  
NEB Golden Gate Assembly Mix 0.5 uL  
H<sub>2</sub>O 7.5 uL  
Total 10 uL

As a negative control (without insert fragment), the following reaction solution should also be prepared each time.

Backbone plasmid vector 0.5 uL  
H<sub>2</sub>O 0.5 uL  
T4 DNA Ligase Buffer (10x) 1 uL  
NEB Golden Gate Assembly Mix 0.5 uL  
H<sub>2</sub>O 7.5 uL  
Total 10 uL

Incubate on a thermal cycler.

37°C 60 min  
60°C 5 min

When introducing the Golden Gate Assembly product into *E. coli* cells, use the following amounts.

Golden Gate Assembly product 2 uL  
*E. coli* competent cell ~50 uL

## **6) Prepare the donor fragment**

The method of preparing the donor fragments depends on the mutation to be introduced. The following is a list of typical cases.

### 6A) Disruption of gene function by indel

This method expects that indel will cause a frameshift, resulting in the appearance of a premature stop codon leading to the destruction of the gene function. Donor fragments are not needed.

### 6B) Deletion of an entire ORF

Prepare a donor fragment (100 bp or more) by PCR (Figure 4), connecting the sequence just outside the ORF to be deleted with two 60-nt oligo DNAs (20 nt of which hybridize to each other).

### 6C) Introduction of nucleotide substitution(s) at specific position(s)

Prepare a donor sequence fragment (~120 bp) containing the nucleotide substitutions by PCR (Figure 5),

using two 70-nt oligo DNAs (20 nt of which hybridize to each other).

6D) Insertion of a gene fragment (fluorescent protein, tag, etc.) at a specific position

Prepare a donor fragment with 35-45 bp homology arms at the gene fragment's 5'-, and 3'-ends by PCR (Figure 6).

### **7) Introduce the genome-editing plasmid and the donor fragment into budding yeast cells**

It is easier to select colonies if you also prepare a negative control to introduce only the genome editing plasmid (without the donor sequence fragment).

#### 7-1) Inoculate the host strain

Inoculate 2 mL of YPAD liquid medium with the budding yeast host strain that has been grown on the agar medium.

YPAD 2 mL

YPAD-dextrose 1800 uL

20% dextrose (glucose) 200 mL

The culture vessel is a disposable 14-mL 2-position tube.

Incubate the culture in a shaker at 25°C overnight.

#### 7-2) Transformation of budding yeast cells

Put 2 mL of budding yeast overnight culture (YPAD medium) into a 2-mL tube.

↓ centrifugation (15,000 rpm, 25°C, 1 min)

Discard the supernatant.

↓

Add

Sterilized water 900 uL

1 M LiAc (lithium acetate) 100 uL.

Suspend the cells well by vortexing.

↓

30°C 45 min (Not too strict, you can make it as long as 90 min)

↓ centrifugation (15,000 rpm, 25°C, 1 min)

Remove 500 uL of the supernatant. (The remaining volume ~500 uL)

↓

Prepare 1.5-mL tubes for the number of samples to be transformed.

↓

Put the following solutions into each tube, and mix and suspend well by vortexing.

(It is convenient to prepare a master mix of LiAc/DTT/carrier DNA)

Yeast cell suspension 50 uL  
1 M LiAc 25 uL  
1 M DTT (dithiothreitol) 25 uL  
carrier DNA 2.5 uL  
Genome-editing plasmid 1 uL  
Donor fragment (PCR product) 46.5 uL

↓

Attach the tip to the P1000 micropipette and cut off the tip end with scissors.

↓

Add ~300 uL of 50% PEG #4000 (polyethylene glycol) by reverse pipetting and suspend the cells well by vortexing.

↓

30°C 90 min

42°C 15 min

↓ centrifugation (5,000 rpm, 25°C, 3 min)

Remove the supernatant slowly with a P1000 micropipette. (Be careful as it is relatively viscous.)

↓

Add 50 uL of SC-Ura-dex (complete synthetic medium without uracil and glucose).

Suspend the cells well by pipetting.

↓

Spread the entire amount of a sample to the SCGal-Ura agar medium. (Use a disposable spreader).

It is convenient to dry and warm up the required number of plates while waiting for the transformation.

↓

Seal the plate with parafilm and incubate at 30°C for 4-7 days.

### **8) Pick the candidate colonies**

Large colonies show very low genome editing efficiency in the case of plasmids that use pGAL1 for both Cas protein and gRNA expression. We select as small colonies as possible.

Select as small colonies as possible, spread each colony in a patch (1 cm x 1 cm) on SCGal-Ura agar medium, seal the plate with parafilm, and incubate at 30°C overnight.

### **9) Check the results of genome editing with PCR**

For deletion of an ORF and insertion of gene fragment, it is easy to confirm the genome editing by PCR. In nucleotide substitutions, PCR assays using primer pairs specific for the pre- and post-editing sequences are required. In the case of confirming indel formation, PCR must be followed by the T7EI assay.

The protocol used in the colony direct PCR of budding yeast is as follows.

[Composition of the reaction mix (20-uL system)]

KOD One 2x master mix 10 uL  
H2O 10 uL

[PCR program]

{ 98°C 0:10  
X°C 0:05 (Use TOYOBO's Tm calculation tool)  
68°C 5 sec/kb } x 30 cycles

### **10) Eliminate the genome-editing plasmid**

Inoculate the clones with positive PCR results into 10 mL of YPAD and incubate at 25-30°C overnight.  
(In some cases, multiple clones can be pooled and cultured together.)

↓

Drop 5 uL of the overnight culture onto a YPD or SC+5FOA plate and spread 3-4 steps using a P1000 tip for single colony isolation.

↓

Patch a single colonies onto a YPD, SC+5FOA, SC-Ura plate and confirm that it does not grow on the SC-Ura plate.

### **11) Perform PCR again to reconfirm the results of genome editing**

The clones after the marker check are subjected to PCR again to confirm that they are indeed clones for which the targeted genome editing is considered to be successful.

### **12) Check the sequence of the PCR product**

The PCR products of the clones with positive PCR results are sequenced to confirm that the sequences of the junction and knock-in portions are as designed. To purify the PCR products for sequencing, we use the following two kits.

#### **Wizard SV Gel and PCR Clean-Up System**

<https://www.promega.jp/products/nucleic-acid-extraction/clean-up-and-concentration/wizard-sv-gel-and-pcr-clean-up-system/?catNum=A9281>

#### **Exo-CIP Rapid PCR Cleanup Kit**

<https://www.nebj.jp/products/detail/2107>