Chapter 24

YeastFab Cloning of Toxic Genes and Protein Expression Optimization in Yeast

Stefan A. Hoffmann

Abstract

YeastFab is a Golden Gate–based cloning standard and parts repository. It is designed for modular, hierarchical assembly of transcription units and multi-gene assemblies for expression in *Saccharomyces cerevisiae*. This makes it a suitable toolbox to optimize the expression strength of heterologous genes in yeast. When cloning heterologous coding sequences into YeastFab vectors, in several cases we have observed toxicity to the cloning host *Escherichia coli*. The provided protocol details how to clone such toxic genes from multiple synthetic DNA fragments while adhering to the YeastFab standard. The presented cloning strategy includes a C-terminal FLAG tag that allows screening for constructs with a desired protein expression in yeast by western blot. The design allows scarlessly removing the tag through a Golden Gate reaction to facilitate cloning of expression constructs with the native, untagged transgene.

Key words Cloning of toxic genes, Heterologous expression, YeastFab, Modular assembly, Removable FLAG tag

1 Introduction

The yeast *Saccharomyces cerevisiae* is one of the most well-studied model organisms, not least thanks to its traditional use in ethanol fermentation and bread making. With substantial knowledge about its physiology, cell biology, and genetics and the availability of a broad toolbox for genetic engineering, it is oftentimes the organism of choice for metabolic engineering [1] or heterologous expression [2]. Developed to facilitate such efforts, YeastFab is both an assembly standard based on Golden Gate cloning and an *S. cerevisiae* genetic parts repository of promoters and terminators [3, 4]. It allows modular construction of transcription units from these parts for expression in yeast and their assembly into multi-gene constructs. YeastFab has been proven to be a useful toolbox to clone heterologous metabolic pathways and optimize metabolite production in yeast [3, 5].

Daniel Schindler (ed.), *Golden Gate Cloning: Methods and Protocols*, Methods in Molecular Biology, vol. 2850,
https://doi.org/10.1007/978-1-0716-4220-7_24,

Following the YeastFab standard, genetic parts, either promoters (PRO), open reading frames (ORF), or terminators (TER), are first cloned into respective entry vectors. This step is also called domestication. In the next level, transcription units are assembled from these parts in so-called POT plasmids (PRO-ORF-TER). The assembly standard results in a cloning scar of only a single base pair between promoter and start codon, and the stop codon and terminator, as the start codon ATG and the stop codon TAG themselves are part of the 4 bp overhang used for Golden Gate assembly. Transcription units can be further assembled into multi-gene constructs.

Cloning can be challenging for recombinant genes with products that are toxic to the cloning host, such as certain nucleases, channel membrane proteins, kinases, or proteins interfering with DNA replication. Readthrough from upstream promoters or cryptic transcription can result in enough gene product being made to exert toxicity on the cloning host *Escherichia coli* even in the absence of dedicated bacterial promoters in front of the gene of interest, as in the YeastFab ORF and POT plasmids. In cases of toxicity to the cloning host, apparent cloning efficiencies will be noticeably low, with cloned genes frequently or always having loss-of-function mutations. This can severely obstruct cloning, both for domestication in an entry vector and for transcription unit assembly in YeastFab. Likewise, the production host can be subject to a gene product's toxicity, which can be tackled by controlling protein expression though use of weak or inducible yeast promoters. In this case, it is desirable to query protein abundance to choose a promoter giving sufficiently high expression without causing a strong growth defect.

Here, we provide a step-by-step protocol to clone a gene with significant toxicity to the cloning host using the YeastFab standard, screen for a suitable yeast promoter with the help of a C-terminal protein tag, and scarlessly remove the tag in a Golden Gate reaction. For reference, an overview of the respective Golden Gate cloning steps is given in Fig. 1.

2 Materials

2.1 Standard Equipment, Consumables, and Software

Standard equipment for molecular cloning (including a plasmid extraction kit and a Qubit fluorometer or similar), SDS-PAGE, and western blot are required. The use of molecular biology software such as SnapGene, Geneious, or Benchling is highly recommended.

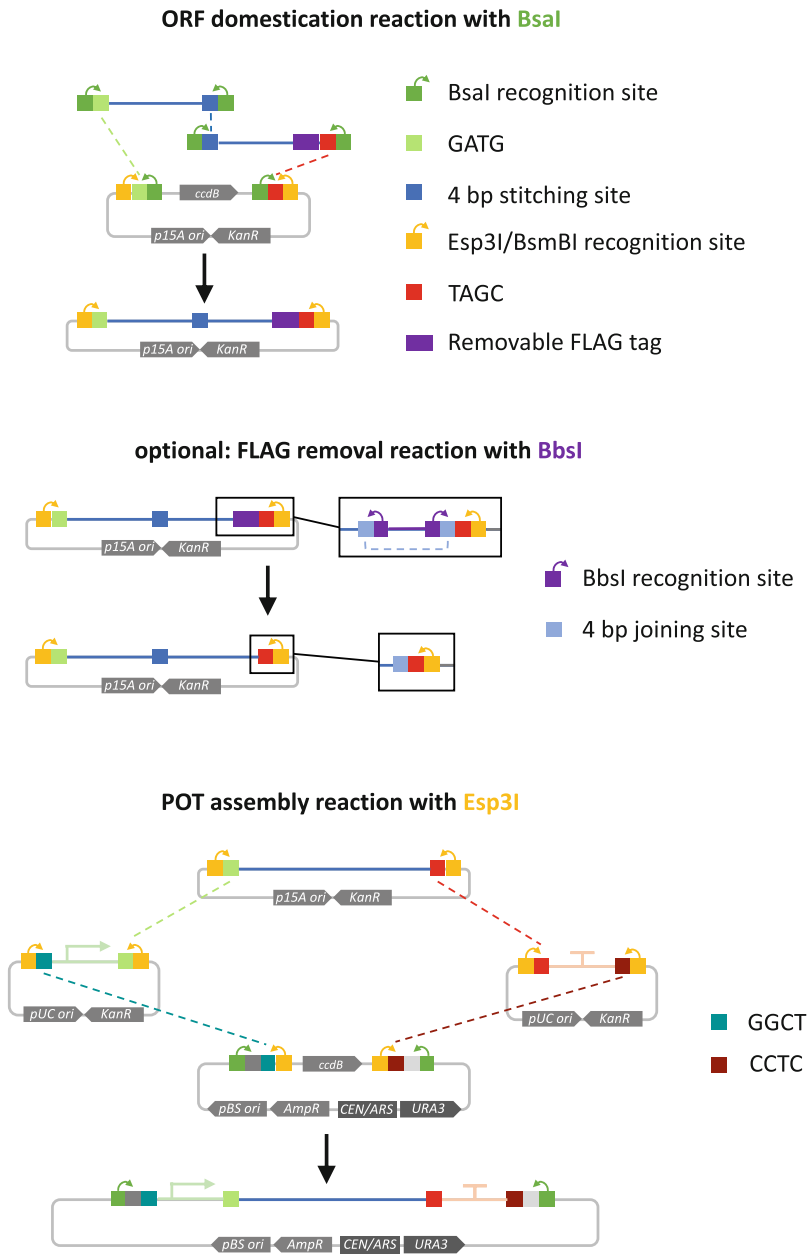

Fig. 1 Overview of Golden Gate cloning steps described. The coding sequence of a large, potentially toxic gene can be domesticated from multiple parts into a low-copy vector compatible with the YeastFab standard. The presented design includes a FLAG tag that can be scarlessly removed from the domesticated coding sequence with a Golden Gate elimination reaction. The coding sequence can then be assembled into a transcription unit for expression in yeast. Promoters and terminators can originate from the YeastFab parts repository or have been cloned according to the same standard

Table 1
Plasmids used in this study

Name	Description	Parental plasmid	References
p15A-Kan-O	Coding sequence low-copy entry vector	HCKan-O	This study
HCKan-P-TDH3	*TDH3* promoter in YeastFab standard	HCKan-P	[3]
HCKan-P-HSP12	*HSP12* promoter in YeastFab standard	HCKan-P	Unpublished
HCKan-P-RPS27B	*RPS27B* promoter in YeastFab standard	HCKan-P	Unpublished
HCKan-P-SOD2	*SOD2* promoter in YeastFab standard	HCKan-P	Unpublished
HCKan-P-ERG1	*ERG1* promoter in YeastFab standard	HCKan-P	Unpublished
HCKan-P-SET5	*SET5* promoter in YeastFab standard	HCKan-P	Unpublished
HCKan-T-CYC3	*CYC3* terminator in YeastFab standard	HCKan-T	Unpublished
POT4	Transcription unit assembly vector	pRS416	[3]

Table 2
DNA oligonucleotides for Sanger sequencing of inserts in HCKan and derivatives

Name	Description	Sequence
HCKan chc Fwd	Sequencing HCKan insert, forward	GATCCTTTGATTTTCTACCG
HCKan chc Rev	Sequencing HCKan insert, reverse	CTCGATAACTCAAAAAATACG

Table 3
Microbial strains used

Species	Strain	Genotype	References
Escherichia coli	DH5α	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20* φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^-	[11]
Saccharomyces cerevisiae	BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	[12]

2.2 Plasmids, Oligonucleotides, and Strains

Plasmids of this protocol are listed in Table 1, used oligonucleotides in Table 2, and strains in Table 3.

2.3 Media

- Luria-Bertani broth (LB): 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, and pH 7.
- Synthetic Complete uracil drop-out medium (SC-Ura): 0.17% (w/v) yeast nitrogen base without amino acids, 0.2% (w/v) yeast synthetic drop-out medium supplements without uracil (Sigma-Aldrich), 0.5% (w/v) ammonium sulfate, and 2% glucose.

- Solid media with the above compositions (LB agar and SC-Ura agar) with additional 2% agar.

2.4 Enzymes

- BsaI-HFv2 (NEB).
- Esp3I.
- BbsI-HF (NEB).
- T4 DNA ligase and buffer.

2.5 Buffers

- Tris-acetate ethylenediaminetetraacetic acid (TAE) buffer: 40 mM Tris base, 20 mM acetic acid, and 1 mM ethylenediaminetetraacetic acid (commercially available as 50× stock solution).
- SDS-PAGE sample buffer: 60 mM Tris-HCl pH 6.8, 4% β-mercaptoethanol, 5% glycerol, 2% sodium dodecyl sulfate, and 0.0025% bromophenol blue.
- SDS-PAGE running buffer: 25 mM Tris base, 192 mM glycine, and 0.1% SDS (commercially available as 10× stock solution).
- Tris-buffered saline Tween (TBST) buffer: 20 mM Tris base, 150 mM NaCl, and 0.1% (w/v) Tween 20 (commercially available as 10× stock solution).

2.6 Other Chemicals

- Kanamycin (50 mg/mL stock in water).
- Carbenicillin or ampicillin (100 mg/mL stock in water).
- Molecular biology grade agarose.
- Glycerol (sterile 50% solution).
- Skim milk powder.

2.7 Consumables Western Blot

- SDS-PAGE gels.
- Nitrocellulose membrane.
- Mouse anti-FLAG antibody M2.
- Mouse anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) or anti-β-actin or anti-α-tubulin.
- Horseradish peroxidase (HRP)-coupled rabbit anti-mouse antibody.
- Enhanced chemiluminescence (ECL) substrate.

3 Methods

3.1 Design of Insert

Here it is assumed that genes to be cloned into the ORF entry vector ("domestication") are obtained as synthetic, linear, double-stranded DNA fragments ("gene fragments"). Typically, those gene fragments are substantially cheaper compared to cloned genes and can be purchased from a variety of DNA synthesis

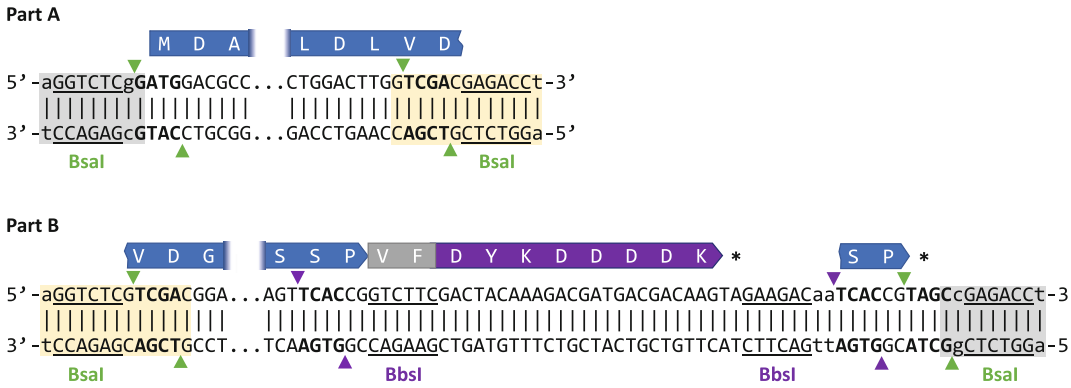

Fig. 2 Design to domesticate vaccinia *D5R* coding sequence gene with a removable FLAG tag from two gene fragments. Recognition sites for type IIs enzymes BsaI and BbsI are underlined, and the cutting sites indicated by matching colors. Translation of the *D5R* coding sequence is shown in blue, of the FLAG tag in purple. Domestication prefix and suffix sequences are highlighted in gray, the sequences used for stitching in yellow

providers. While synthetic gene fragments typically have a maximal length of 2 kb or less, genes larger than that can be cloned as well in a single reaction with a multi-part insert. The gene fragments can be designed to assemble into the full-length gene during the domestication reaction. Further, the design strategy presented here includes a removable FLAG tag as a fully compatible extension of the YeastFab standard. While YeastFab enables easy, modular cloning of transcription units with different promoters and terminators, the FLAG tag allows direct screening for desired protein expression level from those constructs. The tag can be scarlessly removed from the coding sequence through a single Golden Gate reaction. This facilitates cloning of the native, untagged gene without having to amplify and re-domesticate the coding sequence. Most design steps outlined below are easy to achieve with a molecular cloning software such as SnapGene, Geneious, or Benchling.

As an example, the design to clone vaccinia *D5R* with a removable FLAG tag from two gene fragments is illustrated in Fig. 2. It is followed by a generalized procedure detailing the respective design steps of the synthetic DNA fragments for domestication.

1. Obtain the native coding sequence of interest (*see* **Note 1**).
2. Codon optimize the coding sequence for *Saccharomyces cerevisiae*. An open-source codon optimization tool can be found here: cuba.genomefoundry.org/sculpt_a_sequence. Most DNA synthesis providers also provide a free online tool for this purpose but usually require creating an account to access it.
3. If the stop codon is TAA or TGA, change it to TAG.
4. Check for recognition sites for the type IIs restriction enzymes BsaI, BsmBI/Esp3I, and BbsI. Remove all these sites through

synonymous recoding. Preferably change to a codon of a similar frequency in *S. cerevisiae* (*see* **Note 2**).

5. Add the prefix aGGTCTCg**G** directly in front of the **ATG** start codon (5′-aGGTCTCg**GATG**-3′) and the suffix **c**cGAGACCt directly after the **TAG** stop codon (5′-**TAGC**cGAGACCt-3′). The BsaI recognition sites GGTCTC and GAGACC are used for Golden Gate cloning into the entry vector. BsaI cleavage results in four-base overhangs covering the start and stop codons. Identities of nucleotides in lowercase can be changed.
6. For the addition of a removable FLAG tag, add in the sequence shown in bold between the TAG stop codon and the two directly preceding codons (NNNNNN):

NNNNNN-**GTCTTC*GACTACAAAGACGATGACGACAAGTAG*GAAGAC**aa**NNNNNN**-TAG

BbsI recognition sites are underlined, and the FLAG tag including its stop codon is shown in italics. Note that the two codons (**NNNNNN**) now preceding the original stop codon are the same sequence as NNNNNN. This is to create a compatible overhang for scarless removal of the FLAG tag by BbsI.

7. If the coding sequence is too long to be ordered by the supplier of choice as noncloned linear DNA fragment, it can be broken up into smaller pieces to be assembled during the domestication reaction. If this is required, choose a 6 bp stretch (N*NNNN*N) at which to separate the sequence. (Note that the 6 bases NNNNNN here designate a different part of the sequence than in the previous step) The central 4 bp of this stretch will create the overhang for joining the fragments. This 4 bp sequence must not be GATG or TAGC, as these are used as overhangs for cloning into the entry vector. If joining multiple fragments, the 4 bp overhangs of the different fusion sites need to be dissimilar from each other, too. Separate the coding sequence at the chosen position(s). Include the 6 bp stretch N*NNNN*N in both the upstream and the downstream fragments. Add 5′-GAGACCt-3′ at the 3′ end on the upstream fragment and 5′-aGGTCTC-3′ at the 5′ end of the downstream fragment (n_L is the desired sequence left of the 6 bp stretch, n_R is the sequence right of it):
   - Full-length sequence: 5′-n_L-N*NNNN*N-n_R-3′
   - Upstream fragment: 5′-n_L-N*NNNN*N-GAGACCt-3′
   - Downstream fragment: 5′-aGGTCTC-N*NNNN*N-n_R-3′
8. It is recommended to perform the domestication reaction (with BsaI) and the reaction for FLAG tag removal (with BbsI) in silico to check the design. This also provides reference sequences to check sequencing results against.

3.2 Domestication Reaction

In the domestication reaction, the coding sequence of interest is introduced into a YeastFab-compatible entry vector for subsequent POT assembly of transcription units with promoter and terminator. The domestication can be done with a single DNA piece as insert or multiple pieces that have been designed to be concurrently assembled in the Golden Gate reaction. In the example given here (vaccinia *D5R*), the coding sequence is being assembled from two gene fragments. In reactions with more than a single insert, DNA components should be added in equimolar ratios.

Correct clones of vaccinia *D5R* could not be obtained using the standard high-copy YeastFab vector for coding sequence HCKan-O, likely due to cryptic transcription of this gene known to be toxic to *E. coli* [6]. To domesticate toxic genes, a variant of the domestication vector was constructed by swapping the origin of replication for the low-copy p15A origin (p15A-Kan_O). The use of a low-copy domestication vector and growing transformed *E. coli* at 30 °C instead of 37 °C is recommended when toxicity to the cloning host is evident or deemed likely.

1. Add the following reaction components to a PCR tube and mix the components by flicking or brief vortexing and spin the liquid down in a microcentrifuge (*see* **Note 3**).

Component	Volume/amount
Acceptor vector p15A-Kan_O	~80 ng (~50 fmol)
Insert part A	~50 fmol
Insert part B	~50 fmol
10× T4 ligase buffer	1 μL
T4 DNA ligase 400 CEU/μL	0.5 μL
BsaI-HFv2 (NEB) 20 U/μL	0.5 μL
Nuclease-free water	Up to 10 μL

2. Put the PCR tube into a thermocycler with a heated lid and run the following Golden Gate program optimized for multipart assemblies.

Step	Temperature	Cycles	Time
Initial reaction	37 °C	1	1 h
Cycling digest and ligation	37 °C 16 °C	30	5 min 10 min
Final ligation	16 °C	1	20 min
Final digest	37 °C	1	30 min

(continued)

Step	Temperature	Cycles	Time
Inactivation	80 °C	1	5 min
	8 °C		Hold

3. Transform the reaction into a CcdB-sensitive *E. coli* cloning strain. For instance, DH5alpha cells made chemically competent with an RbCl method [7] work well. For their transformation, mix 5 μL of the Golden Gate domestication reaction with 50 μL of the cell suspension of competent cells, incubate for 30 min on ice, heatshock for 45 s at 37 °C, and incubate on ice for another 5 min. Then add 500 μL Luria-Bertani broth (LB) and incubate for 45 min at 37 °C while shaking (*see* **Note 4**).
4. Plate 100 μL of the transformation outgrowth on LB agar with 50 μg/mL kanamycin with a spreader or glass beads. Pellet the remaining cells by centrifugation for 3 min at 6000× *g*. Tip away the supernatant, resuspend the pellet in the remaining liquid (~50 μL), and plate on a second plate of LB agar with kanamycin.
5. Incubate the plates at 30 °C for 2–3 days, after which colonies should be clearly visible. If the insert is toxic to the cloning host, colony sizes might be heterogenous, with correct clones likely forming smaller colonies. Pick colonies of different sizes into 5 mL LB with 50 μg/mL kanamycin each and incubate in a shaking incubator at 30 °C until cultures are dense (typically 1–2 days).
6. Extract plasmid DNA from 4 mL of each of the cultures using a silica column-based plasmid extraction kit according to the manufacturer's instructions. Store the rest of each culture at 4 °C.
7. Perform a test digest on the isolated plasmid DNA to check the insert size. Digest about 200–400 ng of DNA in 10 μL with 5 U of Esp3I in the appropriate buffer. Incubate for about 1 h at 37 °C.
8. Run the digest reactions alongside a suitable DNA ladder on a 1% agarose/TAE gel. A correct clone should give two bands, one from the p15A-Kan_O at 1.8 kb and another one at the size of the assembled insert.
9. Verify candidates by Sanger sequencing. For insert longer than about 1.5 kb, it is recommended to send plasmid DNA for whole plasmid sequencing. Measure DNA concentrations with a fluorescence-based method and prepare samples adjusted to the concentration required by the sequencing service provider (*see* **Note 5**).

10. Compare the sequencing result to the expected sequence. Prepare a cryo-culture according to standard procedure for long-term storage of a correct clone.

3.3 Removal of FLAG Tag

With the presented design, a single Golden Gate reaction allows to generate tag-free versions of domesticated coding sequences. This obviates the need to PCR amplify or re-order and re-clone the coding sequence. The type IIs enzyme used for this purpose (BbsI) is orthogonal to the ones used by the YeastFab standard (BsaI, Esp3I/BsmBI).

1. Add the following reaction components to a PCR tube and mix the components by flicking or brief vortexing and spin the liquid down in a microcentrifuge.

Component	Volume/amount
Coding sequence with FLAG in p15A-Kan_O	~20 ng
10× T4 ligase buffer	1 μL
T4 DNA ligase 400 CEU/μL	0.5 μL
BbsI-HF (NEB) 10 U/μL	0.5 μL
Nuclease-free water	Up to 10 μL

2. Put the PCR tube into a thermocycler with a heated lid and run the Golden Gate program as in Subheading 3.2, **step 2** (*see* **Note 6**).
3. Transform into *E. coli* as in Subheadings 3.2, **steps 3** and **4**.
4. Incubate the plates for 2 days at 30 °C and pick two or more colonies into 5 mL LB with 50 μg/mL kanamycin, again making sure to include small colonies in case of size heterogeneity.
5. Incubate in a shaking incubator at 30 °C until cultures are dense and isolate plasmid DNA from 4 mL of fresh cultures, storing cultures at 4 °C until sequence verification.
6. Perform a test digest on the isolated plasmid DNA to check the insert size. Digest about 200–400 ng of DNA in 10 μL with 5 U of Esp3I in the appropriate buffer. Incubate for about 1 h at 37 °C.
7. Run the digest reactions alongside a suitable DNA ladder on a 1% agarose/TAE gel. A correct clone should give two bands, one from the backbone and one from the insert, like in the domestication reaction (*see* **Note 7**).
8. For sequence confirmation, send prepared plasmid DNA for Sanger sequencing with the primer "HCKan chc Rev," covering the 3′ part of the coding sequence, or send for whole

plasmid sequencing for larger inserts. The sequencing is to confirm removal of the FLAG tag and that the gene has not been mutationally altered. Prepare a cryo-culture according to standard procedure for long-term storage of a correct clone.

3.4 Assembly of Transcription Units

Once the coding sequence has been successfully domesticated in p15A-Kan_O, it can be assembled in POT reactions with promoters and terminators according to the YeastFab standard. Facile construction of different expression constructs including the protein tag allows screening for desired expression behavior, balancing protein expression strength and potential fitness defects. Combinations of promoters and terminators giving desirable behavior can be used to construct transcriptional units without FLAG tag from the p15A-Kan_O construct with the tag-less coding sequence created in "Removal of FLAG tag" using the same protocol.

In the given example, the domesticated *D5R* coding sequence is assembled in the YeastFab standard in POT4 with a *CYC3* terminator and different promoters. Promoters ranging from very strong to fairly weak were chosen (reported gene transcript counts [8] in their endogenous context in brackets). Namely, the 500 bp upstream sequences of *TDH3* (15,909), *HSP12* (56), *RPS27B* (5356), *SOD2* (267), *ERG1* (307), and *SET5* (60) are tested for expression of *D5R*. However, transcription is known to be highly context dependent [9]. For instance, we have previously observed the 500 bp upstream of *HSP12* to be a rather strong promoter (unpublished) in the YeastFab reporter system [4].

1. Adjust the concentration of DNA parts to be assembled with the domesticated coding sequence, promoters in HCKan-P, terminators in HCKan-T, and the POT vector of choice (*see* **Note 8**), to 50 ng/μL each. This allows setting up multiple reactions with different components without having to adjust volumes.
2. Add the following reaction components to a PCR tube.

Component	Volume/Amount
Promoter in HCKan-P 50 ng/μL	1 μL
Terminator in HCKan-T 50 ng/μL	1 μL
POT vector 50 ng/μL	2 μL
Coding sequence in p15A-Kan_O	~30 fmol
10× T4 ligase buffer	1 μL
T4 DNA ligase 400 CEU/μL	0.5 μL
Esp3I 10 U/μL	0.5 μL
Nuclease-free water	Up to 10 μL

3. Proceed with the Golden Gate reaction and subsequent transformation into *E. coli*, as done in Subheadings 3.2, **steps 3, 4** and **5**, but plate on LB agar with 100 μg/mL carbenicillin or ampicillin.
4. Incubate the plates for 2 days at 30 °C and pick several colonies into 5 mL LB with 100 μg/mL carbenicillin or ampicillin, again making sure to include small colonies in case of size heterogeneity.
5. Incubate in a shaking incubator at 30 °C until cultures are dense and isolate plasmid DNA from 2 mL of fresh cultures, storing cultures at 4 °C until sequence verification.
6. Perform a test digest of the isolated plasmid DNA to check the insert size. Digest about 200–400 ng of DNA in 10 μL with 10 U of BsaI-HFv2. Incubate for about 1 h at 37 °C.
7. Run the digest reactions alongside a suitable DNA ladder on a 1% agarose/TAE gel. A correct clone should give two bands, one at 4.9 kb from the POT vector backbone, and another one at the size corresponding to the coding sequence plus the promoter length (500 bp in YeastFab standard parts) and terminator length (200 bp in YeastFab standard parts).
8. For sequence confirmation, send plasmid DNA of clones with correct digest patterns for whole plasmid sequencing. Prepare a cryo-culture according to standard procedure for long-term storage of a correct clone.

3.5 Assaying Protein Expression in Yeast

Transcription units with FLAG tag assembled in a POT vector can be screened for their protein expression. Constructs are first transformed into yeast. Transformed strains are grown to prepare SDS-PAGE samples, which are then assayed for protein expression using a western blotting procedure. The steps are briefly outlined here. Consult protocols describing SDS-PAGE and western blotting for detailed instructions on the techniques (e.g., [10]).

Assessing the *D5R* constructs with different promoters by western blot showed a range of expression strengths of D5 protein, covering strong (*pTDH3*, *pRPS27B*), medium (*pHSP12*), weak (*pSOD2*, *pERG1*), and very weak or absent (*pSET5*) expression (Fig. 3). None of the constructs imparted noticeable toxicity on the yeast host.

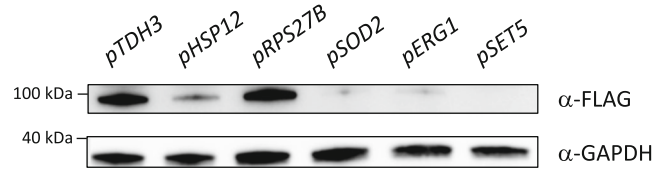

Fig. 3 Anti-FLAG tag western blot against D5-FLAG expressed in yeast from assembled *D5R* transcriptional units with indicated promoters. GAPDH was used as loading control

1. From a dense overnight culture of *S. cerevisiae* BY4742 in yeast extract peptone dextrose medium (YPD), inoculate a fresh culture 1:50 in YPD. For every transformation, a culture volume of 5 mL is needed. Grow the culture for 4 h at 30 °C with orbital shaking.
2. Pellet the cells (4000× *g* for 5 min) and discard the supernatant. Wash with half the original volume of sterile distilled water by resuspending and pelleting. Repeat the wash step once.
3. Resuspend the pellet in 1 mL 100 mM lithium acetate and pellet in a microcentrifuge tube at 10,000× *g* for 15 s. Remove the supernatant by pipetting and resuspend pellet in 100 mM lithium acetate with 50 μL for every 5 mL starting culture.
4. Denature 10 mg/mL salmon sperm DNA by incubating 5 min at 100 °C and placing on ice immediately after. For each transformation, 12.5 μL is needed.
5. For each transformation, to 50 μL of the cell suspension in 100 mM lithium acetate, add 266.67 μL of 50% polyethylene glycol 3350, 40 μL of 1 M lithium acetate, 12.5 μL 10 mg/mL denatured salmon sperm DNA, and about 500 ng of the POT construct. Make up to a total volume 400 μL with sterile distilled water.
6. Vortex mixture for 1 min. Incubate shaking at 30 °C for 1 h.
7. Heat shock for 20 min at 42 °C in a dry bath or water bath.
8. Pellet cells (4000× *g* for 3 min), remove supernatant, and resuspend cells in 200 μL sterile distilled water. Spread 50 μL of the cell suspension on SC-Ura agar using sterile glass beads or a cell spreader.
9. Incubate plates at 30 °C for 2–3 days until colonies appear. Plates with colonies can be stored for up to a month at 4 °C.
10. Inoculate 10 mL of SC-Ura for each transformation from single colonies and incubate shaking overnight at 30 °C.
11. From dense overnight cultures, inoculate fresh cultures 1:50 in 10 mL SC-Ura. Grow the culture for 4 h at 30 °C with orbital shaking.
12. Measure the optical density at 600 nm for each culture. Collect the volume equivalent to 1 optical density unit for each; 1 optical density unit equals 1 mL of a culture at an optical density of 1.
13. Pellet cells (10,000× *g* for 5 min), remove supernatant, and resuspend cells in 500 μL cold distilled water. Pellet again, remove supernatant by pipetting, and resuspend 200 μL 100 mM NaOH.
14. Incubate for 10 min at room temperature for alkaline lysis of the cell walls. Pellet cells again (6000× *g* for 5 min), remove

supernatant by pipetting, and resuspend each in 50 μL SDS-PAGE sample buffer. Samples can be stored at −20 °C until use.

15. Run 10 μL of each sample on a gradient SDS-PAGE gel for 40 min at 200 V along with a pre-stained protein ladder.
16. Blot the gel onto a nitrocellulose or polyvinylidene fluoride membrane with a method of your choice.
17. Assess successful blotting by staining membrane with Ponceau S. After staining, wash away excess dye with water and image the membrane. Afterward, wash with TBST until the membrane is mostly destained.
18. Block membrane by incubation in 5% skim milk powder in TBST for 1 h at room temperature.
19. Incubate with mouse anti-FLAG M2 antibody (Sigma), wash membrane three times with TBST, and incubate with a secondary HRP-coupled anti-mouse antibody. Wash again three times and develop with ECL.
20. As loading control, choose a suitable housekeeping protein (e.g., GAPDH, β-actin, α-tubulin) with a molecular weight different enough to be clearly separated from the protein of interest. Develop with a primary mouse antibody and a secondary HRP-coupled anti-mouse antibody analogous to **step 18**.

4 Notes

1. Coding sequences can for instances be retrieved from files from NCBI's GenBank (www.ncbi.nlm.nih.gov/genbank), a vast database containing annotated nucleotide sequences.
2. The *Saccharomyces cerevisiae* nuclear genome codon usage table can be found here: https://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4932&aa=1&style=N
3. A DNA molarity calculator can facilitate calculation of required DNA masses from desired molar amounts and fragment lengths. Such a tool can for instance be found here: www.bioline.com/media/calculator/01_07.html
4. Other protocols for competent cell preparation and transformation can be used, including commercially available kits for preparation or ready-made competent *E. coli*. In each case, a transformation outgrowth step must be included to allow cells to build resistance against kanamycin.
5. To verify the sequence of shorter inserts, Sanger sequencing can be used. Suitable sequencing primers are "HCKan chc Fwd" and "HCKan chc Rev." Both are priming on the plasmid

backbone of all HCKan vectors and the p15A-Kan_O derivative near the cloning site, pointing toward the insert.

6. For the elimination reaction a shorter Golden Gate reaction scheme with a reduced number of cycles can also be used.
7. Toxic genes are frequently inactivated through insertions of transposons. This will be detectable through an increased size of the insert.
8. The choice of the POT vector is relevant if multi-gene assemblies are to be carried out subsequently and informs the position of the transcriptional unit in those higher-order assemblies [3].

Acknowledgments

The author would like to thank Yizhi Cai for supporting this work. Plasmids with promoters in YeastFab standard were a gift from Junbiao Dai, while HCKan-T-CYC3 was a gift from Yizhi Cai.

References

1. Nielsen J, Larsson C, van Maris A, Pronk J (2013) Metabolic engineering of yeast for production of fuels and chemicals. Curr Opin Biotechnol 24(3):398–404. https://doi.org/10.1016/j.copbio.2013.03.023
2. Romanos MA, Scorer CA, Clare JJ (1992) Foreign gene expression in yeast: a review. Yeast 8(6):423–488. https://doi.org/10.1002/yea.320080602
3. Guo Y, Dong J, Zhou T, Auxillos J, Li T, Zhang W, Wang L, Shen Y, Luo Y, Zheng Y, Lin J, Chen GQ, Wu Q, Cai Y, Dai J (2015) YeastFab: the design and construction of standard biological parts for metabolic engineering in Saccharomyces cerevisiae. Nucleic Acids Res 43(13):e88. https://doi.org/10.1093/nar/gkv464
4. Garcia-Ruiz E, Auxillos J, Li T, Dai J, Cai Y (2018) YeastFab: high-throughput genetic parts construction, measurement, and pathway engineering in yeast. Methods Enzymol 608:277–306. https://doi.org/10.1016/bs.mie.2018.05.003
5. Zhou Y, Li G, Dong J, Xing XH, Dai J, Zhang C (2018) MiYA, an efficient machine-learning workflow in conjunction with the YeastFab assembly strategy for combinatorial optimization of heterologous metabolic pathways in Saccharomyces cerevisiae. Metab Eng 47:294–302. https://doi.org/10.1016/j.ymben.2018.03.020
6. Evans E, Klemperer N, Ghosh R, Traktman P (1995) The vaccinia virus D5 protein, which is required for DNA replication, is a nucleic acid-independent nucleoside triphosphatase. J Virol 69(9):5353–5361. https://doi.org/10.1128/JVI.69.9.5353-5361.1995
7. Green R, Rogers EJ (2013) Transformation of chemically competent E. Coli. Methods Enzymol 529:329–336. https://doi.org/10.1016/B978-0-12-418687-3.00028-8
8. Lipson D, Raz T, Kieu A, Jones DR, Giladi E, Thayer E, Thompson JF, Letovsky S, Milos P, Causey M (2009) Quantification of the yeast transcriptome by single-molecule sequencing. Nat Biotechnol 27(7):652–658. https://doi.org/10.1038/nbt.1551
9. Brooks AN, Hughes AL, Clauder-Munster S, Mitchell LA, Boeke JD, Steinmetz LM (2022) Transcriptional neighborhoods regulate transcript isoform lengths and expression levels. Science 375(6584):1000–1005. https://doi.org/10.1126/science.abg0162
10. Hnasko TS, Hnasko RM (2015) The Western blot. Methods Mol Biol 1318:87–96. https://doi.org/10.1007/978-1-4939-2742-5_9
11. Grant SG, Jessee J, Bloom FR, Hanahan D (1990) Differential plasmid rescue from transgenic mouse DNAs into Escherichia coli methylation-restriction mutants. Proc Natl Acad Sci USA 87(12):4645–4649. https://doi.org/10.1073/pnas.87.12.4645

12. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14(2):115–132. https://doi.org/10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2