Published in final edited form as:

Yeast. 2015 December ; 32(12): 711–720. doi:10.1002/yea.3098.

New Vectors for Simple and Streamlined CRISPR-Cas9 Genome Editing in *Saccharomyces cerevisiae*

Marian F. Laughery¹, Tierra Hunter¹, Alexander Brown¹, James Hoopes¹, Travis Ostbye¹, Taven Shumaker¹, and John J. Wyrick^{1,2,*}

¹School of Molecular Biosciences, Washington State University, Pullman, WA 99164, USA

²Center for Reproductive Biology, Washington State University, Pullman, WA 99164, USA

Abstract

CRISPR-Cas9 technology is an important tool for genome editing because the Cas9 endonuclease can induce targeted DNA double-strand breaks. Targeting of the DNA break is typically controlled by a single-guide RNA (sgRNA) – a chimeric RNA containing a structural segment important for Cas9 binding and a 20mer guide sequence that hybridizes to the genomic DNA target. Previous studies have demonstrated that CRISPR-Cas9 technology can be used for efficient, marker-free genome editing in *S. cerevisiae*. However, introducing the 20mer guide sequence into yeast sgRNA expression vectors often requires cloning procedures that are complex, time-consuming, and/or expensive. To simplify this process, we have developed a new sgRNA expression cassette with internal restriction enzyme sites that permit rapid, directional cloning of 20mer guide sequences. Here we describe a flexible set of vectors based on this design for cloning and expressing sgRNAs (and Cas9) in yeast using different selectable markers. We anticipate that the Cas9-sgRNA expression vector with the *URA3* selectable marker (pML104) will be particularly useful for genome editing in yeast, since the Cas9 machinery can be easily removed by counter-selection using 5-fluoroorotic acid (5-FOA) following successful genome editing. The availability of new vectors that simplify and streamline the technical steps required for guide sequence cloning should help accelerate the use of CRISPR-Cas9 technology in yeast genome editing.

Keywords

Saccharomyces cerevisiae; Cas9; CRISPR; plasmids; guide RNA; genome editing

Introduction

Genome editing technologies are important tools for altering genes and genomic regions. The critical factor for efficient genome editing is the induction of a DNA double strand break near the targeted genomic locus (Carroll, 2014). DNA double strand breaks greatly enhance DNA replacement by homologous recombination and also stimulate error-prone nonhomologous end joining (NHEJ).

* Correspondence to: John J. Wyrick, School of Molecular Biosciences, Biotechnology Life Sciences 241, Washington State University, Pullman, WA 99164 USA. jwyrick@vetmed.wsu.edu.

So far, the simplest and most flexible method for targeting double strand breaks is the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system (Doudna and Charpentier, 2014; Hsu, et al., 2014). The most common CRISPR systems use the Cas9 protein from *Streptococcus pyogenes*, which catalyzes DNA breaks at genomic sites that match a 20mer guide segment of the Cas9-bound CRISPR RNA (crRNA). Cas9 catalyzes the hybridization of the guide RNA to its target genomic locus and subsequent DNA cleavage as long as a protospacer-adjacent motif (PAM) is also present in the genomic sequence immediately 3' to the target site (Deltcheva, et al., 2011; Jinek, et al., 2012). The *S. pyogenes* Cas9 requires a 5'-NGG-3' PAM sequence for efficient cleavage, although 5'-NAG-3' PAM sites can be cleaved with less efficiency (Hsu, et al., 2014; Hsu, et al., 2013; Jiang, et al., 2013). In its host bacteria, Cas9 binds a pair of hybridized RNAs: the crRNA containing the 20mer guide sequence and the trans-activating CRISPR RNA (tracrRNA; (Deltcheva, et al., 2011; Jinek, et al., 2012)). A key advance was the discovery that a hybrid 'single guide' RNA (sgRNA) that fuses the crRNA guide sequence to the tracrRNA can be used for efficient Cas9 targeting (Jinek, et al., 2012) in a plethora of different organisms (Doudna and Charpentier, 2014; Hsu, et al., 2014; Sternberg and Doudna, 2015).

Budding yeast (*Saccharomyces cerevisiae*) was one of the first organisms in which CRISPR-Cas9 gene editing was successfully demonstrated (DiCarlo, et al., 2013). Unlike mammalian cells, Cas9-induced breaks in yeast generate relatively few random mutations (DiCarlo, et al., 2013), presumably due to the refractory nature of the blunt DNA breaks to NHEJ repair in yeast (Bahmed, et al., 2011; Hegde and Klein, 2000; Herrmann, et al., 1998) and the efficiency of the yeast recombination machinery. Instead, yeast CRISPR-Cas9 systems primarily stimulate recombination with a template sequence, typically either a long oligonucleotide or PCR product that is co-transformed with the Cas9 and sgRNA expressing vector(s). A key advantage of the yeast system is that CRISPR-Cas9 DNA cleavage can also be used to select for daughter cells with the correct genome alteration, thus enabling 'marker-free' genome editing (DiCarlo, et al., 2013). This method requires that the template contain mutations in the sgRNA target site or adjacent PAM sequence so that daughter cells with the targeted genome alteration are resistant to subsequent Cas9 cleavage. Constitutive expression of Cas9 and sgRNA selects against the remaining unedited yeast cells due to the continual induction of toxic DNA breaks.

The original description of CRISPR-Cas9 gene editing in yeast targeted single genomic loci using two plasmids: one expressing Cas9 and the other expressing the sgRNA (DiCarlo, et al., 2013). Recent reports have described more advanced CRISPR-Cas9 vector systems that use one- or two-plasmid systems to target up to five genomic loci in yeast simultaneously (Bao, et al., 2015; Horwitz, et al., 2015; Jakociunas, et al., 2015; Ryan, et al., 2014). A drawback to each of these published vectors is the relative difficulty and expense in introducing the 20mer guide sequence into the sgRNA (or crRNA array) expression cassette. In the original description of yeast CRISPR-Cas9 gene editing (DiCarlo, et al., 2013), the sgRNA expression plasmid was generated through at least two distinct PCR amplifications and/or Gibson assembly. A second report (Bao, et al., 2015) synthesized large gene blocks containing the assembled guide RNA sequences, which were inserted into plasmids using the Golden Gate assembly method (Engler, et al., 2009; Engler, et al., 2008). A third report

(Jakociunas, et al., 2015) used PCR amplification, PCR product circularization, and uracil-specific excision reaction (USER) cloning (Bitinaite, et al., 2007; Jensen, et al., 2014) to assemble the guide RNA expression cassette. A fourth report (Ryan, et al., 2014) did not fully describe the cloning strategy and vectors that were used, but was rated to be more labor-intensive than the previous two methods described above (Jakociunas, et al., 2015). A fifth report generated gRNA cassettes through two consecutive PCR reactions and used the gRNA PCR product directly in a transformation reaction (Horwitz, et al., 2015); however, the editing efficiency was decreased relative to gRNA cassette plasmids and the transformations were conducted in strains with the Cas9 gene previously integrated in the genome, which is not desirable for many genome editing applications. Some investigators have even resorted to synthesizing (and cloning) an entire gene block containing the sgRNA expression cassette (>400 bp) for each individual guide sequence (Zhang, et al., 2014).

Here we describe a new set of vectors for CRISPR-Cas9 genome editing in *Saccharomyces cerevisiae*. A key advantage of these vectors is that the design of the sgRNA expression cassette greatly simplifies cloning of the 20mer guide sequence. Unique BclI and SmaI restriction enzymes enable simple directional cloning of hybridized oligos containing the user-designed 20mer guide sequence and the 5' end of the structural region of the sgRNA. Furthermore, these oligonucleotide sequences can be readily designed using new online web tools specifically developed for this purpose. Additionally, the development of a single *URA3* vector with both the sgRNA expression cassette and Cas9 gene should enable the rapid removal of the Cas9 machinery following successful genome editing. We anticipate that the new vectors will facilitate the widespread use of CRISPR-Cas9 gene editing in yeast.

Materials and Methods

Plasmid Construction

To construct the guide RNA expression vector pT040, a 499 bp gene block (gWY001) was synthesized (Integrated DNA Technologies) containing the *SNR52* promoter, unique BclI and SmaI sites, the 3' end of the single guide RNA (sgRNA) and *SUP4* terminator (Figure 1). The design of the promoter, sgRNA, and terminator was based on a previously published yeast sgRNA expression cassette (DiCarlo, et al., 2013). The gene block was PCR amplified with primers OWY139 and OWY140. The resulting PCR product was digested with SacII and EagI and cloned into a similarly digested pCT3 plasmid (Thompson, et al., 1993) to generate pT040 (Figure 2).

The Cas9 overexpression vector (pJH001) was constructed by site-directed mutagenesis and subcloning the Cas9 construct to a yeast high-copy expression vector. A modified version of the QuikChange site-directed mutagenesis protocol (Agilent Technologies) was used to restore the D10 and H840 residues in the pTDH3-dCas9 vector (Gilbert, et al., 2013) using primers OWY193/OWY194 and OWY195/OWY196, respectively, to create plasmid pT022. An EcoRI-XbaI fragment containing the *TDH3* promoter (pTDH3), Cas9 open reading frame, SV40 nuclear localization sequence (SV40 NLS), and *ADHI* terminator was subcloned into similarly digested pRSII425 plasmid to generate pJH001 (Figure 2).

The pML104 and pML107 vectors, which contain both Cas9 and sgRNA expression cassette (Figure 2), were constructed as follows. Four BclI sites in the Cas9 open reading frame were mutated sequentially by site-directed mutagenesis using primers OAB001-OAB008 to create a 'BclI-free' Cas9 construct. The gWY001 gene block containing the sgRNA expression cassette was cloned into the *URA3* 2-micron plasmid pRSII426 using EagI/SacII restriction sites. Next, the 'BclI-free' Cas9 construct was cloned into this backbone using XhoI/EcoRI sites to create pML104. Finally, the entire Cas9-gWY001 cassette was cloned into the pRSII425 backbone at the EcoRI/SacII restriction sites to create pML107.

Plasmid construction was confirmed by sequencing and/or restriction enzyme digestion. Primer sequences are available upon request.

Guide RNA Cloning Protocol

The guide RNA expression plasmids (i.e., pT040, pML104, and pML107) were constructed to have unique BclI and SmaI restriction sites to facilitate guide RNA cloning. The plasmid DNA was prepared from a dam- *Escherichia coli* (*E. coli*) strain (New England Biolabs). Plasmids were typically digested with SmaI (New England Biolabs) overnight at 25°C followed by heat inactivation at 65°C for 20 minutes. BclI (New England BioLabs) was subsequently added to the digestion reaction and incubated for a minimum of two hours at 50°C. Digested plasmids were purified by gel electrophoresis and isolated from gel fragments using the Zymoclean Gel DNA recovery kit (Zymo Research). A stock of digested sgRNA expression plasmid was used for cloning custom 20mer guide sequences (see below).

For *TRP1* gene targeting, oligonucleotides OWY251 and OWY252 were hybridized at a concentration of 3 μ M in a solution of 1 $\times$ T4 DNA ligase Buffer (50mM Tris-HCl, 10mM MgCl₂, 1mM ATP, 10mM DTT, pH 7.5). Hybridization was performed in a thermocycler (MJ Research). Reactions were initially held at 95° C for six minutes, and then decreased 1° C per one minute cycle for seventy cycles to reach a final temperature of 25° C. Hybridized oligonucleotide substrates containing the 20mer guide sequence and 5' end of the sgRNA were then directly ligated into cut sgRNA expression plasmids (e.g., pT040, pML104, or pML107) typically overnight at 16° C. The hybridized the nucleotide insert was added at a 40-100 fold molar ratio to the cut vector. The ligation reaction was transformed into Z-competent *E. coli* (Zymo Research), and plasmid DNA from the resulting colonies was isolated and sequenced using a T3 primer to confirm *TRP1* guide sequence insertion into the sgRNA expression cassette.

Streamlined Yeast Transformation Protocol for CRISPR Genome Editing

Yeast transformations for CRISPR-Cas9 gene editing were performed using a modified version of the one-step yeast transformation protocol (Chen, et al., 1992). For two-plasmid (i.e., pT040 and pJH001) CRISPR-Cas9 experiments, yeast cell cultures were grown overnight in synthetic complete leucine dropout media (SC-LEU) to maintain selection for the pJH001 Cas9 plasmid. The following day, 125-250 μ L of yeast cells were pelleted by centrifugation, supernatants were removed, and the cells were resuspended in One Step Buffer (OSB, 0.2N LiOAc, 30% PEG 3350, 100 mM DTT). Yeast were incubated with

~50-250 ng of pT041 plasmid (sgRNA expression plasmid targeting *TRP1*) along with 12.5-125 pmoles of the heterologous donor OWY256 single-stranded oligonucleotide (ssOligo) or hybridized OWY255/OWY256 double-stranded oligonucleotides (dsOligos), or 1 µg of purified PCR cassette. In addition, 12.5-25 µg of salmon sperm DNA was added as single stranded carrier DNA to increase transformation efficiency. Transformation reactions were incubated for 30 minutes at approximately 45° C and directly plated on synthetic complete (SC) leucine and uracil dropout media (SC-LEU-URA). Plates were incubated four days at 30° C before colony counting.

A similar transformation protocol was used for the single-plasmid (i.e., pML104 or pML107) CRISPR-Cas9 experiments, except yeast cultures were grown overnight in Yeast Extract Peptone Dextrose media (YPD), and the resulting transformation reactions were plated on either SC-URA or SC-LEU plates.

Validation of *trp1* Mutants

To test for *trp1* mutants following CRISPR-Cas9 transformation, transformants were streaked for isolation onto plates containing the same selective medium (e.g., SC-LEU-URA). Colonies from these plates were then patched or replica plated first to SC-TRP plates, and then to plates containing tryptophan. Plates were incubated at 30° C for three days or until obvious growth had occurred.

For sequence analysis, genomic DNA extraction was performed as first described by Looke and colleagues (Looke, et al., 2011). Swipes of isolated colonies were resuspended in 200 mM LiAc 1% SDS and incubated at 70° C for five minutes. Three volumes of 100% ethanol were added and the genomic DNA was precipitated by centrifugation. The genomic DNA was washed with 70% ethanol, resuspended, and then a second ethanol precipitation was performed to further purify the genomic DNA.

PCR was performed on the resuspended genomic DNA to amplify the targeted *trp1* locus using flanking primers OWY285 and OWY286. PCR products were purified using a DNA Clean & Concentrator-5 Kit (Zymo Research) and used as templates for DNA sequencing with the internal OWY268 *trp1* primer.

Development of Online Tools for CRISPR-Cas9 Guide Sequence Cloning

We developed online tools to search for unique guide RNA target sites in the yeast genome and design oligonucleotides for cloning the guide sequences into the sgRNA expression vectors. We used a published list of unique guide RNA targets in the yeast genome (DiCarlo, et al., 2013). Perl scripts were developed to identify and display all possible guide RNA targets within a user-selected gene or genomic region. Additionally, these tools display the oligonucleotide sequences needed for hybridization and cloning into the sgRNA expression vectors. These tools are available at the website: <http://wyrickbioinfo2.smb.wsu.edu/crispr.html>.

Results and Discussion

Design of an sgRNA expression cassette for efficient 20mer guide sequence cloning

The rate-limiting step for CRISPR-Cas9 genome editing in yeast is often cloning the 20mer guide sequence into its expression vector. Inserting the guide sequence into the appropriate location within the sgRNA expression cassette can require multiple time-consuming (and often expensive) cloning steps. This is not an intrinsic limitation of the CRISPR-Cas9 system itself, but a design limitation of currently available vectors. For example, a previously published yeast sgRNA expression cassette (DiCarlo, et al., 2013), consisting of the *SNR52* promoter, sgRNA sequence, and *SUP4* terminator, lacks unique restriction sites for guide sequence cloning.

We designed a 499 bp sgRNA expression cassette with unique *BclI* and *SwaI* restriction enzyme sites to enable rapid, directional cloning of 20mer guide sequences (Figure 1). The *BclI* restriction site is located at the 3' end of the *SNR52* promoter (p*SNR52*) and generates a 5' GATC overhang when digested; the *SwaI* restriction site is located within the structural segment of the sgRNA and generates a blunt end when digested. This permits efficient ligation of short hybridized oligos designed with compatible 5' GATC overhang and blunt ends (Figure 1). Ligation of the hybridized oligos regenerates the 3' end of the *SNR52* promoter and inserts the 20mer guide sequence immediately downstream of this promoter. Because the *SwaI* site is located within the sgRNA, it is important for the hybridized oligos to include after the 20mer guide sequence an additional 13 bp of sequence containing the 5' end of the sgRNA (i.e., 5'-GTTTTAGAGCTAG-3'; see Figure 1). Online tools to help with the design of these oligonucleotide sequences are available at <http://wyrickbioinfo2.smb.wsu.edu/crispr.html>.

Cas9 genome editing in yeast using the new sgRNA expression cassette

The pT040 sgRNA expression vector (Figure 2A) was created by cloning the new sgRNA expression cassette into the pCT3 backbone (Thompson, et al., 1993). This backbone was chosen because it lacks *SwaI* and *BclI* sites, which would interfere with guide sequence cloning. Our practice is to generate large stocks of pT040 sgRNA expression vector that have been digested with *BclI* and *SwaI* enzymes and subsequently gel purified. Importantly, the vector DNA must be purified from a dam- *E. coli* strain, as Dam methylation blocks *BclI* digestion. Once a stock of cut sgRNA vector has been prepared, unique guide sequences can be cloned by hybridization of the user-designed oligonucleotides and ligation into the cut sgRNA vector. The oligonucleotides do not need to be phosphorylated or purified for efficient ligation. Using this approach, guide RNA sequences for CRISPR gene editing can be easily cloned and verified in just a few days.

We tested the genome editing efficiency of the pT040 vector by targeting the yeast *TRP1* gene. Oligonucleotides OWY251 and OWY252, which contain a 20mer guide sequence targeting the *TRP1* open reading frame (Figure 1), were hybridized and ligated into pT040 to create plasmid pT041. The pT041 vector was transformed into a BY4741 strain containing pTDH3-Cas9 (pT022), a low-copy yeast centromere plasmid with a *LEU2* selectable marker that expresses Cas9. The transformations also contained a 90mer oligonucleotide template

(OWY255/OWY256) to introduce a single nucleotide deletion and two nucleotide substitutions within the *TRP1* gene. These mutations simultaneously eliminate the PAM sequence adjacent to the guide RNA target and introduce a frame shift mutation to truncate the Trp1 protein (Figure 3). We selected for transformants that grew on media lacking uracil and leucine, indicating they contained vectors expressing both the *TRP1* guide RNA and Cas9 enzyme.

While the empty sgRNA expression vector (pT040) could be transformed efficiently into the Cas9 expressing yeast strain, the *TRP1* sgRNA expression vector (pT041) yielded few transformants in the absence of an oligonucleotide template with the targeted mutation (Table 1, top panel). These results indicate efficient selection against ‘unedited’ transformants, likely due to the continual induction of toxic DNA breaks by the constitutively expressed Cas9 and guide RNA constructs. We sequenced one of the few background transformants that was *trp*⁻ and found a single base pair deletion of a cytosine nucleotide immediately adjacent to the Cas9 cut site (Figure 3 and data not shown). This mutation simultaneously disrupts the *TRP1* gene and the guide sequence target, rendering the cells resistant to Cas9 cutting. Presumably this mutation arose from error-prone NHEJ repair, which occurs at low frequency in yeast.

Co-transformation of the single-stranded (OWY256) or double-stranded (OWY255/OWY256) oligonucleotide template (ssOligo or dsOligo, respectively) increased the median number of transformants nearly 20-30 fold (Table 1, top panel). The increase in transformation efficiency likely reflects efficient homologous recombination with the oligonucleotide template. Indeed, over 98% of these transformants were *trp*⁻ and sequencing of two transformants confirmed the introduction of the targeted mutation. Experiments co-transforming a PCR product (i.e., *TRP1* knockout cassette) as the template also yielded successful genome editing, although the targeted editing efficiency was decreased (data not shown). This may be due to the relatively low molar concentration of the PCR template in the transformation.

To investigate if the gene-editing efficacy can be improved by increasing Cas9 protein level, we repeated the *TRP1* genome editing experiments using a BY4741 yeast strain expressing a high-copy Cas9 expression vector (pJH001; Figure 2B). Again, relatively few background colonies were observed in the transformation of the *TRP1*-sgRNA vector alone (Table 1, bottom panel). However, co-transformation with an oligonucleotide template yielded more transformants with the high copy Cas9 expression vector, particularly when using the double-stranded oligonucleotide template (Table 1, bottom panel). Importantly, 100% of the tested transformants were *trp*⁻, and sequencing of a few colonies confirmed the introduction of the targeted *trp1* mutation by homologous recombination with the oligonucleotide template. It is not clear if the increased genome editing efficiency in the high-copy Cas9 expression strain is due to increased Cas9 activity or an overall increase in transformation efficiency in this strain.

All of these results were obtained using a simple, low-efficiency yeast transformation protocol (see Materials and Methods), which typically requires only about an hour to perform (Chen, et al., 1992). Because of the efficiency of Cas9 genome editing, lengthy

high-efficiency yeast transformation protocols are not necessary. Occasionally, we observed that a few *TRP*⁺ cells would arise from a *trp*[−] colony when selecting for growth on media lacking tryptophan. The number of unedited cells was very small, and they are presumably present either within the colony itself (i.e., the colony contained a small fraction of unedited cells) or in an adjacent region of the plate that was mistakenly included when the colony was picked. No unedited cells were found after streaking the transformant colony for further growth on selective SC-LEU-URA plates; hence, we subsequently adopted this as our standard practice (see Materials and Methods).

New vectors for single-plasmid CRISPR-Cas9 genome editing

To simplify the genome editing procedure, we constructed vectors in which Cas9 and the sgRNA expression cassette are present in a single *URA3* (pML104) or *LEU2* (pML107) high-copy vector (Figure 2C and D). In order for these vectors to be compatible with directional cloning of guide sequences into the sgRNA expression cassette, we introduced silent mutations to eliminate four *Bcl*I cut sites within the Cas9 open reading frame. With these vectors, a single co-transformation reaction of the vector (with inserted guide sequence) and the oligonucleotide template can generate the targeted mutation. In addition to streamlining the transformation procedure, a single vector is more easily removed from the yeast strain following successful genome editing. This is particularly true for the pML104 vector, which can be efficiently removed by counter-selection with 5-FOA.

We tested the efficacy of the pML104 and pML107 vectors in CRISPR-Cas9 genome editing by targeting the yeast *TRP1* locus using the strategy described in Figure 3. As shown in Table 2, many transformants were obtained with the empty vector (i.e., pML104 or pML107), but the transformation frequency decreased precipitously for vectors containing the active *TRP1* guide RNA (i.e., pML105 or pML106). These data indicate that the single-plasmid vectors were able to efficiently select against unedited transformants in the absence of a template for recombination. Co-transformation of the double stranded oligonucleotide template with the *TRP1*-sgRNA expression vector yielded a higher number of transformants, nearly all of which were *trp*[−] (Table 2). We sequenced ten of the *trp*[−] colonies, and found that 100% had the targeted mutation. These data confirm that CRISPR-Cas9 genome editing can be efficiently performed using vectors pML104 and pML107. The *URA*⁺ transformants obtained from the *TRP1*-sgRNA vector (pML105) + dsOligo transformation were a mix of very small and normal-sized colonies. The normal-sized colonies were nearly all *trp*[−] (Table 2); however, the very small colonies had a lower *trp*[−] frequency (78% *trp*[−] out of 23 colonies tested; data not shown), suggesting that some of the very small colonies escaped Cas9 selection against unedited transformants. Hence, it is recommended to screen normal-sized transformant colonies unless the targeted mutation itself could cause a slow-growth phenotype.

In addition to targeting *TRP1*, our lab has used these vectors (Figure 2) to create point mutations or deletions in promoters (e.g., *RAD2* and *RFX1* promoters) and gene coding regions (e.g., *ESAI*, *RAD4*, *RNRI*, and *STH1*). A number of these are essential genes, which are challenging to mutate using other marker-free methods such as *delitto perfetto* (Storici and Resnick, 2006). A 90mer oligonucleotide template was used to create the desired

mutation, while simultaneously introducing mutations (or deletions) in the PAM motif and/or guide RNA target. Typically, the mutations were located in the center of the 90mer template, with at least 30-40 nucleotides of flanking sequence on each end. Our lab has also used these vectors to insert PCR constructs containing *lacZ* or *HIS3* reporter genes (e.g., at Flo8) and C-terminal tags (e.g., at *MAG1*, *RAD1*, and *SPT16*). In summary, these CRISPR-Cas9 vectors can be used to make a wide variety of substitution, deletion, or insertion mutations in the yeast genome.

Conclusions

We have shown that vectors containing our simplified sgRNA expression cassette can be used to rapidly perform marker-free genome editing in yeast. The design of unique BclI and SmaI restriction enzyme sites within the sgRNA cassette make the process of cloning guide sequences simple, rapid, and inexpensive. Indeed, once a stock of pre-cut vector has been prepared, the only direct expenses associated with guide sequence cloning are the synthesis of two short oligonucleotides, the T4 DNA ligase reaction, and the cost of sequencing to ensure guide RNA insertion. CRISPR-Cas9 genome editing is a transformational technology. The availability of these new vectors, which simplify and streamline the technical steps required for guide sequence cloning, should help accelerate the adoption of this technology within the yeast scientific community.

Acknowledgements

We thank Russell Wilson for expert technical assistance and John Choi for computer help. We are grateful to Dr. Peng Mao and Amelia Hodges for helpful comments and suggestions. Arati Bhattarai, Amelia Hodges, Dr. Peng Mao, Nicole Reinsch, Linh Tran, and other Wyrick lab members have used and tested the CRISPR-Cas9 vectors. Vectors pT040, pJH001, pML104, and pML107 have been deposited at Addgene (www.addgene.org). This work was supported in part by grant number ES002614 from the National Institute of Environmental Health Sciences (NIEHS). The contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIEHS or NIH.

References

- Bahmed K, Seth A, Nitiss KC, Nitiss JL. End-processing during nonhomologous end-joining: a role for exonuclease 1. *Nucleic Acids Res.* 2011; 39:970–8. [PubMed: 20935051]
- Bao Z, Xiao H, Liang J, Zhang L, Xiong X, Sun N, Si T, Zhao H. Homology-Integrated CRISPR-Cas (HI-CRISPR) System for One-Step Multigene Disruption in *Saccharomyces cerevisiae*. *ACS Synth Biol.* 2015; 4:585–94. [PubMed: 25207793]
- Bitinaite J, Rubino M, Varma KH, Schildkraut I, Vaisvila R, Vaiskunaite R. USER friendly DNA engineering and cloning method by uracil excision. *Nucleic Acids Res.* 2007; 35:1992–2002. [PubMed: 17341463]
- Carroll D. Genome engineering with targetable nucleases. *Annu Rev Biochem.* 2014; 83:409–39. [PubMed: 24606144]
- Chen DC, Yang BC, Kuo TT. One-step transformation of yeast in stationary phase. *Curr Genet.* 1992; 21:83–4. [PubMed: 1735128]
- Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E. CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature.* 2011; 471:602–7. [PubMed: 21455174]
- DiCarlo JE, Norville JE, Mali P, Rios X, Aach J, Church GM. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Res.* 2013; 41:4336–43. [PubMed: 23460208]
- Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science.* 2014; 346:1258096. [PubMed: 25430774]

- Engler C, Gruetzner R, Kandzia R, Marillonnet S. Golden gate shuffling: a onepot DNA shuffling method based on type IIs restriction enzymes. *PLoS One*. 2009; 4:e5553. [PubMed: 19436741]
- Engler C, Kandzia R, Marillonnet S. A one pot, one step, precision cloning method with high throughput capability. *PLoS One*. 2008; 3:e3647. [PubMed: 18985154]
- Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, Stern-Ginossar N, Brandman O, Whitehead EH, Doudna JA, Lim WA, Weissman JS, Qi LS. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. 2013; 154:442–51. [PubMed: 23849981]
- Hegde V, Klein H. Requirement for the SRS2 DNA helicase gene in nonhomologous end joining in yeast. *Nucleic Acids Res*. 2000; 28:2779–83. [PubMed: 10908335]
- Herrmann G, Lindahl T, Schar P. *Saccharomyces cerevisiae* LIF1: a function involved in DNA double-strand break repair related to mammalian XRCC4. *EMBO J*. 1998; 17:4188–98. [PubMed: 9670033]
- Horwitz AA, Walter JM, Schubert MG, Kung SH, Hawkins K, Platt DM, Hernday AD, Mahatdejkul-Meadows T, Szeto W, Chandran SS, Newman JD. Efficient Multiplexed Integration of Synergistic Alleles and Metabolic Pathways in Yeasts via CRISPR-Cas. *Cell Systems*. 2015; 1:1–9.
- Hsu PD, Lander ES, Zhang F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell*. 2014; 157:1262–78. [PubMed: 24906146]
- Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, Cradick TJ, Marraffini LA, Bao G, Zhang F. DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*. 2013; 31:827–32. [PubMed: 23873081]
- Jakociunas T, Bonde I, Herrgard M, Harrison SJ, Kristensen M, Pedersen LE, Jensen MK, Keasling JD. Multiplex metabolic pathway engineering using CRISPR/Cas9 in *Saccharomyces cerevisiae*. *Metab Eng*. 2015; 28:213–22. [PubMed: 25638686]
- Jensen NB, Strucko T, Kildegaard KR, David F, Maury J, Mortensen UH, Forster J, Nielsen J, Borodina I. EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. *FEMS Yeast Res*. 2014; 14:238–48. [PubMed: 24151867]
- Jiang W, Bikard D, Cox D, Zhang F, Marraffini LA. RNA-guided editing of bacterial genomes using CRISPR-Cas systems. *Nat Biotechnol*. 2013; 31:233–9. [PubMed: 23360965]
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*. 2012; 337:816–21. [PubMed: 22745249]
- Looke M, Kristjuhan K, Kristjuhan A. Extraction of genomic DNA from yeasts for PCR-based applications. *Biotechniques*. 2011; 50:325–8. [PubMed: 21548894]
- Ryan OW, Skerker JM, Maurer MJ, Li X, Tsai JC, Poddar S, Lee ME, DeLoache W, Dueber JE, Arkin AP, Cate JH. Selection of chromosomal DNA libraries using a multiplex CRISPR system. *Elife*. 2014; 3
- Sternberg SH, Doudna JA. Expanding the Biologist's Toolkit with CRISPRCas9. *Mol Cell*. 2015; 58:568–574. [PubMed: 26000842]
- Storici F, Resnick MA. The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. *Methods Enzymol*. 2006; 409:329–45. [PubMed: 16793410]
- Thompson CM, Koleske AJ, Chao DM, Young RA. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell*. 1993; 73:1361–75. [PubMed: 8324825]
- Zhang GC, Kong II, Kim H, Liu JJ, Cate JH, Jin YS. Construction of a quadruple auxotrophic mutant of an industrial polyploid *saccharomyces cerevisiae* strain by using RNA-guided Cas9 nuclease. *Appl Environ Microbiol*. 2014; 80:7694–701. [PubMed: 25281382]

Figure 1.

Design of new guide RNA expression cassette for rapid cloning of 20mer guide sequences. Unique BclI and SwaI sites enable efficient cloning of any 20mer guide RNA targeting sequence into the single guide RNA (sgRNA) expression cassette. A plasmid with the guide RNA expression cassette is linearized by digestion with BclI and SwaI enzymes. Oligonucleotides are designed to contain a compatible GATC overhang, a 20mer guide sequence (in this case targeting the yeast *TRP1* gene), and the 5' end of the structural segment of the sgRNA. The hybridized oligonucleotides are ligated into the digested plasmid, yielding the final complete sgRNA expression cassette. The asterisk indicates that BclI cutting is blocked by Dam methylation.

Figure 2.

Maps of new plasmids for CRISPR-Cas9 genome editing in *Saccharomyces cerevisiae*. (A and B) Vectors for two-plasmid system of CRISPR-Cas9 gene editing. pT040 has the guide RNA expression cassette with unique BclI-SmaI restriction enzyme sites for cloning the 20mer guide sequence. pJH001 is a high-copy Cas9 expression vector. (C and D) *URA3* and *LEU2* vectors for one-plasmid system of CRISPR-Cas9 gene editing. Each vector contains the Cas9 gene and the guide RNA expression cassette with unique BclI-SmaI restriction enzyme sites for cloning the 20mer guide sequence (see Figure 1). The asterisk indicates that BclI cutting is blocked by Dam methylation.

Targeting the yeast *TRP1* gene for CRISPR-Cas9 gene editing. The genomic DNA (gDNA) region targeted by the guide sequence is indicated, along with the location of the Cas9 cut site. The *TRP1* start codon is underlined. A portion of the 90mer oligonucleotide template with the targeted *TRP1* mutation is shown. Recombination between the gDNA and template eliminates the PAM sequence adjacent to the Cas9 cut site and introduces an early frame shift in the *TRP1* gene.

Table 1

Transformation and *TRP1* editing efficiency using pT040 sgRNA vector and low or high copy Cas9 expression vector

	Transformants (Median)	Percentage <i>trp</i> -
<i>Low-copy Cas9 expression vector^a</i>		
empty sgRNA vector ^b	175	–
<i>TRP1</i> -sgRNA vector ^c alone	1	56% (16 ^d)
<i>TRP1</i> -sgRNA vector + ssOligo template	20	96% (23)
<i>TRP1</i> -sgRNA vector + dsOligo template	29	98% (53)
<i>High-copy Cas9 expression vector^e</i>		
empty sgRNA vector	~1400	0% (24)
empty sgRNA vector + dsOligo template	~1600	0% (72)
<i>TRP1</i> -sgRNA vector alone	3	83% (35)
<i>TRP1</i> -sgRNA vector + ssOligo template	39	100% (60)
<i>TRP1</i> -sgRNA vector + dsOligo template	215	100% (24)

^a pT022

^b pT040

^c pT041

^d Number of transformant colonies tested

^e pJH001

Table 2Transformation and *TRP1* editing efficiency using pML104 and pML107 vectors

	Transformants (Median)	Percentage <i>trp</i> -
<i>pML104</i>		
empty sgRNA cassette ^a + dsOligo template	>3500	0% (24 ^b)
<i>TRP1</i> -sgRNA vector ^c alone	6	23% (13)
<i>TRP1</i> -sgRNA vector + dsOligo template	86	97% (36 ^d)
<i>pML107</i>		
empty sgRNA cassette ^e + dsOligo template	570	0% (32)
<i>TRP1</i> -sgRNA vector alone ^f	2	— ^g
<i>TRP1</i> -sgRNA vector + dsOligo template	16	96% (27)

^a pML104 (and other vectors) also contain Cas9^b Number of transformant colonies tested^c pML105^d Only includes data from normal-sized transformant colonies (see text for details)^e pML107^f pML106^g Insufficient colonies to accurately measure the percentage of *trp*- colonies